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13. ABSTRACT (Maximum 200) To facilitate the search for mutations in neurofibromatosis 2 (NF2), we have defined the exon-intron boundaries for the NF2 gene. PCR amplification of each exon in the NF2 gene combined with single strand conformation polymorphism (SSCP) analysis has identified a number of germline mutations in NF2 families and somatic alterations in sporadic vestibular schwannomas and meningiomas. Thus inactivation of the NF2 gene product merlin is a common feature underlying both inherited and sporadic forms of the NF2 related tumors. In order to understand the normal function of the NF2 protein merlin, we have generated a panel of monoclonal antibodies specific for merlin. Using indirect immunofluorescence and one of the monoclonal antibodies, we have for the first time visualized endogenous merlin and localized it to the motile regions, such as leading or ruffling edged, in human cells. Membrane ruffles are particularly seen at the leading edges of motile cells where they are believed to be required for directed cell migration. It is likely that merlin is important for cell movement, shape or communication. Investigation of these processes in Schwann cells and meningeal cells that are the targets of NF2 mutations may represent a fruitful avenue to elucidate the mechanism underlying the tumor suppressor function of merlin.					
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FOREWORD

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INTRODUCTION

Neurofibromatosis 2 (NF2) (a.k.a. central neurofibromatosis, bilateral acoustic neurofibromatosis) is a severe inherited disorder genetically and clinically distinct from the more common neurofibromatosis 1 (NF1) or von Recklinghausen neurofibromatosis (peripheral neurofibromatosis)¹⁻³. NF2 is inherited in an autosomal dominant fashion with high penetrance, and affects approximately 1 in 40,000 individuals. About one half of NF2 cases are thought to be new mutations as they reveal no family history. The hallmark of NF2 is the bilateral occurrence of vestibular schwannomas (formerly called acoustic neuromas), benign, slow-growing tumors on the vestibular branch of the 8th cranial nerve¹⁻³. However, other tumors, particularly meningiomas, spinal schwannomas, and ependymomas, are also frequent. In addition, many patients develop posterior capsular opacities⁴. The same tumor types that occur as multiple growths in NF2 also occur as sporadic, solitary tumors in the general population. Persons with NF2 generally develop symptoms of 8th nerve dysfunction, including deafness and balance disorder, in early adulthood. In exceptional cases, onset occurs in childhood or is delayed until the fifth or sixth decade. The tumors of NF2 are histologically benign, but their anatomical locations make management difficult. While they cause functional loss by compressing adjacent nerves, surgical removal often risks permanent nerve damage. Consequently, NF2 patients suffer great morbidity and mortality, having significantly a shortened average life span.

In 1986-1987, we discovered that DNA markers on chromosome 22 revealed frequent loss of heterozygosity in both the tumors of NF2 and in their sporadic counterparts⁵⁻⁷. We predicted that the *NF2* gene lay on chromosome 22, and that its function was that of a "tumor suppressor" that acted normally to prevent tumor growth. In the tumor suppressor model⁸, it was supposed that both copies of this gene in a single cell must be inactivated for that cell to escape this step in growth control and consequently to form a tumor. In sporadic tumors, both copies of the gene would be inactivated by somatic mutation events, including in some cases chromosomal deletions. The rarity of coincident events occurring in a single cell would explain the typical absence of multiple tumors in non-inherited cases. In NF2 families, the defect was presumed to be a copy of the tumor suppressor gene already inactivated by a mutation transmitted in the germline. Cells in NF2 patients would then require only a single somatic mutational event to inactivate the remaining gene copy, explaining the frequent occurrence of multiple tumors in these inherited cases. A number of subsequent studies of both the inherited and sporadic tumors supported this tumor suppressor hypothesis⁹⁻¹⁴, but validation of the model required a direct

- ▶ demonstration that the inherited defect causing NF2 mapped in the region of chromosome 22 most frequently altered in the tumors.

We obtained this validation in a family study of a single, extremely large, extended NF2 pedigree^{15,16}. The disorder was tracked through multiple generations, and its pattern of inheritance was correlated with the inheritance of polymorphic DNA markers from chromosome 22. The results revealed that the NF2 defect was transmitted within a segment of the central portion of the chromosome 22 long arm, in band 22q12. Subsequent analyses of this and other smaller NF2 families progressively refined the localization of the genetic defect which appears to account for all cases of the disorder^{17,18}. In early 1993, we successfully identified the *NF2* tumor suppressor gene based on its map position using a location cloning approach¹⁹. The predicted protein product of this gene showed a strong similarity to a family of cytoskeleton-associated proteins and was named merlin (for moesin-ezrin-radixin-like protein). Members of this family, whose prototype is protein 4.1 of the erythrocyte, are conserved throughout mammalian species and may function in a number of roles, including mediating communication between the extracellular milieu and the cytoskeleton by acting as a linker between integral membrane proteins and the scaffolding proteins of the filamentous submembrane lattice²⁰. The identification of merlin as a tumor suppressor was confirmed independently by Rouleau and co-workers, who named the protein schwannomin²¹.

The *NF2* gene spans 110 kb, comprising 16 constitutive exons and one alternatively spliced exon and producing mRNAs in three different size ranges, ~7 kb, ~4.4 kb and ~2.6 kb that encode at least two major alternative forms of merlin^{19,21-23}. The alternatively spliced exon 16 alters the C-terminus of the protein, replacing 16 amino acids with 11 novel residues^{22,23}. Additional alternative splices predicting other minor species have also been described^{24,25}. The mouse homologue, which maps to Chr 11, is similarly alternatively spliced and predicts a protein that is 98% identical to human merlin²⁶⁻²⁸. The initial identification of merlin as the *NF2* suppressor was based on 4 non-overlapping interstitial deletions in 4 independent NF2 patients¹⁹.

The primary goal of this Cooperative Agreement effort was to perform a detailed analysis of mutations in the neurofibromatosis 2 (NF2) gene. With the NF2 gene in hand, it became possible to determine how defects at this locus lead to the formation of both familial and sporadic tumors. A large number of mutations have been defined in NF2 patients, and in both sporadic and familial schwannomas and meningiomas^{21-23,29-40}

In this Cooperative agreement we intended:

1. To examine the germline and somatic mutations in NF2-derived tumors.
2. To delineate the range of mutations in sporadic tumors corresponding to the types seen in NF2 such as vestibular schwannomas and meningiomas.
3. To explore whether mutations at the NF2 locus also occur in sporadic tumors not normally associated with NF2, particularly the tumors that display loss of heterozygosity on chromosome 22q such as colon carcinoma.
4. To generate a panel of monoclonal antibodies against merlin in order to determine the expression of this protein in different cells of the nervous system.

We have been extremely successful in all these specific aims and a summary of the work accomplished is reported here.

BODY

Characterization of the NF2 gene

To facilitate mutation searching, we generated flanking intron sequence for each exon and designed genomic PCR assays for single strand conformational polymorphism (SSCP) scanning (*Jacoby et al., Hum Mol Genet, 1994*). We also identified an alternatively spliced exon that removes the final 16 residues of merlin, and replaces them with 11 novel residues, creating a second major isoform of merlin (*Bianchi et al., Nature Genet 1994; Jacoby et al., Hum Mol Genet, 1994*). The two isoforms are also present in the mouse gene which encodes a protein 98% identical to human merlin (*Haase et al., Hum Mol Genet 1994*). We have mapped mouse *Nf-2* to Chr 11, near the mouse *Nefh* and *Lif* genes (*Haase et al., Hum Mol Genet 1994*), a region containing no mouse mutant locus that produces a phenotype similar to NF2. In man, *NEFH* and *LIF* flank *NF2*, indicating a region of synteny conservation in the mouse spanning this segment of human 22q12.

Mutations affecting the NF2 gene

Unlike the search for mutations in *NF1*, the analysis of the *NF2* gene has revealed a plethora of germline and somatic mutations affecting merlin expression. However, the same investigations have shown a remarkably low rate of benign polymorphism in this gene. In our original report of the gene's discovery, we reported independent NF2 patients with a ~40 kb genomic deletion that proved to remove exons 2-4 and a smaller 3-4 kb deletion that removed exons 13 and 14¹⁹. In two meningioma tumors from NF2 patients, we found frameshifts caused

- by a 4 bp deletion in exon 14 and a 1 bp deletion in exon 3, respectively. Both of these alterations also proved to be germline mutations.

In a detailed analysis of vestibular schwannomas, we used the exon PCR assays to perform single-strand conformation polymorphism analysis of DNA from 30 sporadic and 8 NF2-derived tumors. Of 60 alleles scanned, 30 showed mutations affecting expression of merlin, indicating that inactivation of this locus is a common feature underlying both inherited and sporadic forms of schwannoma (*Jacoby et al. Hum Mol Genet 1994*). The majority of the somatic changes were small deletions or insertions that created a frameshift leading to a premature stop codon, or that altered splicing. The most frequent germline alterations were point mutations that introduced premature stop codons or disrupted consensus splicing signals, except 4 missense mutations that affect highly conserved residues and one in-frame deletion that removes a single amino acid residue (*Jacoby et al. Hum Mol Genet 1994; MacCollin et al., AJHG 1994*). We have also detected mutations in vestibular schwannomas using an RT-PCR approach to reveal two deletions extending between exons 3 and 4 and exons 4 and 5, respectively, as well as several cases of missing single exons that might be due to either splicing mutations or genomic deletions (*Bianchi et al., Nature Genet 1994*). Continued analysis of a second cohort of 58 vestibular schwannomas has revealed an additional 62 mutations that fit the same pattern as those described above. Essentially, inactivating mutations have been detected in all exons except exons 16 and 17, encoding the alternative C-termini, and are relatively evenly distributed across the first 15 exons with no outstanding hot-spots (*Jacoby et al Genes Chromosomes and Cancer 1996*).

Besides germline mutations in NF2, and somatic alterations in vestibular schwannomas, we have also examined other NF2-associated and non-NF2-associated tumors for mutations. In a collaborative study we analyzed the entire coding region of NF2 in 70 sporadic meningiomas, identifying 43 mutations in 41 patients. As in schwannomas, these alterations resulted predominantly in immediate truncation, splicing abnormalities or altered reading frame. All the mutations were in the first 13 exons, with no evidence for a hot-spot³⁶. Presence of NF2 mutations in these tumors was usually associated with loss of heterozygosity (LOH) on chromosome 22, supporting the view that the NF2 gene represents the purported meningioma locus on this chromosome. However, NF2 mutations were far more frequent in fibroblastic and transitional than in meningothelial meningiomas, suggesting that there are different molecular mechanisms of meningioma pathogenesis. We have also detected NF2 mutations in 2 spinal schwannomas and in a spinal ependymoma, both tumor types seen in NF2 patients (*Rubio et al. Cancer Res 1994*). In a scan of 30 astrocytomas, we detected no changes in NF2. Thus, despite

- frequent LOH on chromosome 22 in these non-NF2 tumors, merlin does not appear to be the tumor suppressor involved (*Rubio et al. Cancer Res 1994*). We have observed similar results in colon carcinomas (*Rustgi et al., 1995*).

Generation of Monoclonal Antibody for merlin

To complement our polyclonal antisera for merlin, we have generated monoclonal antibodies (with support from this co-operative agreement) using different segments of merlin expressed as GST fusion protein. In our initial attempt, we injected the amino-terminal segment of merlin that spans residues 1-332. Four different monoclonals were obtained and none of them turned out to be specific for merlin. Our subsequent attempts using the carboxy-terminus of merlin spanning residues 308-590 (specific for isoform 2) or spanning residues 308-579 have produced three different monoclonal antibodies 1C4, 4B7, and 12F7 specific for merlin. For monoclonal antibody production, four mice were injected with 50 µg of thrombin cleaved merlin (GST-MER). After the final boost the best responders were sacrificed, spleens were removed and fused to Sp2 myeloma cells. Fusions and the selection were carried out as described (Harlow and Lane, 1988). Class and subclass were determined using an isotyping kit (Amersham). The mAb 1C4 was found to be an IgG1, κ; 4B7 was IgG2a, κ and 12F7 was IgG1, κ. As mAb 1C4 has turned out to be quite useful for the endogenous visualization of merlin, we have used this antibody extensively for the work described below.

Expression of merlin in different cell types

We have examined the expression of merlin in a variety of human cell lines by Western blot analyses using rabbit polyclonal antisera as well as mAb1C4. Merlin migrates as an ~66 kD in various human cell lines including adult fibroblasts, fetal fibroblasts (MRC-5), glioma cells (H238), HeLa cells as well as in two primary meningioma cell lines established from sporadic meningiomas without *NF2* mutations (MN12 and MN27). The latter tumors belong to that subset of sporadic meningiomas thought to be due to genetic lesions in an as yet unidentified locus rather than the mutations in the *NF2* gene^{35, 36}. Other meningioma cell lines from the tumors of NF2 patients with known mutations lack the expression of merlin.

Cellular localization of endogenous merlin

The similarity between merlin and the ERMs suggests that it may also associate with both membrane and cytoskeletal structures. Initial immunocytochemical studies of exogenous merlin overexpressed in COS-7 cells transfected with *NF2* cDNA have supported the localization of the protein at or near the cell membrane⁴¹. However, there has been no report of detection and localization of endogenous merlin, expressed at physiological levels. To determine the cellular

- ▶ localization of endogenous merlin, we have screened various cell lines with all of our anti-merlin antibodies by indirect immunofluorescence. In cell lines that express merlin as detected by Western blots, none of the affinity eluted polyclonal antibodies is able to detect endogenous merlin in paraformaldehyde fixed and permeabilized cells. By contrast, the 1C4 monoclonal antibody detects endogenous merlin in human fetal and adult fibroblasts as well as in various primary meningioma cells. The localization is predominantly in the motile parts of the cells such as ruffling and leading edges (*Gonzalez-Agosti, Xu et al, 1996*).⁴². No staining of these structures was detected in meningioma cells which do not express merlin. The mAb 1C4, fails to detect the endogenous protein in cells such as glioma (H238), and HeLa, cells in which expression is detected by immunoblot. This is a common phenomenon observed with the ERM proteins⁴³, perhaps due to more diffuse localization or to masking of epitopes in certain cell types. Similarly, no staining is observed with the rat Schwann cell line (S-16), rat newborn fibroblasts (RNF), primary rat Schwann cells and NIH-3T3 cells.

Merlin and F-actin co-localize within leading lamellae

The ERM proteins co-localize with actin in surface microvilli and membrane ruffles in some cell types^{43, 44}. A binding site for F-actin in the carboxy-terminal 34 amino acids of ezrin is highly conserved in moesin and radixin, but is not present in merlin⁴⁵. To determine whether merlin and F-actin also co-localize within the cell, we performed double staining in primary meningioma cells (MN12) employing mAb 1C4 to detect merlin, and rhodamine phalloidin to detect F-actin simultaneously. These two proteins localize to the motile sites of the cells mainly at the ruffling edges. Merlin did not co-localize with F-actin at the stress fibers (*Gonzalez-Agosti, Xu et al, 1996*).

Comparison of subcellular localization of merlin to ERM proteins

The ERM proteins localize to the ruffling membrane, microvilli, and filopodia among many other sites reported in various cell types. To determine whether merlin localizes to these structures, we compared their pattern of endogenous expression in human fibroblasts (RD136) and in meningioma cells (MN12). In both these cell types, merlin localizes to the motile regions. The anti-ERM antibodies reveal only weak staining of the human fibroblasts, precluding direct comparison with merlin. However, in MN12 meningioma cells, moesin localizes to the microvilli, and ezrin localizes to the filopodia, at the sites where two cells contact each other. Anti-radixin antibody consistently shows no significant staining. Thus, the localization of the ERM proteins moesin and ezrin in MN12 cells is quite distinct from that of endogenous merlin which localizes to the ruffling edges but never to filopodia or microvilli (*Gonzalez-Agosti, Xu et al, 1996*).

Tumor formation due to inactivation of merlin probably results from perturbation of a signal transduction pathway that induces the target cell to grow and divide. The normal function of this pathway could involve such tasks as monitoring the state of the cytoskeleton or passing information from membrane signals simultaneously to the nucleus and the cytoskeleton. Merlin's localization suggests that it may be involved in the regulation or process of membrane ruffling, a common event observed in many cell types, often in response to certain extracellular factors. Membrane ruffles are particularly seen at the leading edges of motile cells where they are believed to be required for directed cell migration. Several previously characterized signal transducing molecules are implicated in the membrane ruffling response⁴⁶. Studies have demonstrated the role for small GTP-binding proteins *Rho* and *Rac* in the formation of the membrane ruffles and the reorganization of the cytoskeleton^{47, 48}. ERM family members have been reported to co-localize with *Rho* in a number of cellular locations, including TPA-induced membrane ruffles in MDCK2 cells⁴⁸.

Whether *Rho* and *Rac* play a role in NF2 tumor formation remains to be determined, but it is likely that merlin is important for cell movement, shape or communication. Investigation of these processes in the Schwann cells and meningeal cells that are the targets of *NF2* mutations may represent a fruitful avenue to elucidate the mechanism underlying the tumor suppressor function of merlin.

Expression of merlin in human tissues:

We have analyzed the expression of merlin in different areas of normal human brain where it is expressed as a 66 kD protein. Immunohistochemistry studies have revealed wide expression of merlin in different sections of brain. Merlin is expressed in almost all astrocytes, subpopulations of neurons. Expression is either weak or not detectable in ependymal cells, schwann cells and arachnoid (manuscript in preparation). We have not observed the expression of merlin by either Western blot or immunohistochemistry in any of the vestibular schwannomas analyzed so far. However, all three ERM family members are expressed in these tumors (Ramesh, unpublished). A detailed study on the expression of merlin in other human tissues is underway.

CONCLUSIONS

Our approach for the mutational analysis of the *NF2* gene in patients with *NF2*, as well as in sporadic vestibular schwannomas and meningiomas have revealed a high frequency of mutations in this gene. Majority of these mutations involve small deletions or insertions resulting

- in frame shift, point mutations creating stop codons, or interference with normal splicing. Although, thus far, the number of missense events found to affect merlin is low, these events may be important in elucidating the function of merlin protein and its domain. Perhaps the most immediate result of this effort is the ability to provide pre symptomatic screening to at-risk family members, improving diagnostic certainty and reducing the need for costly radiographic and audiologic screening. For many family members this testing will serve to supplement a already ongoing screening program and should pose minimal additional psychological burdens.

As part of this co-operative agreement, we have generated a panel of monoclonal antibodies for merlin, which serve as a valuable reagent in understanding the normal cellular function of merlin and its role as a tumor suppressor. These antibodies are quite useful in scanning the expression of merlin in schwannomas and meningiomas. While most tumors would not be expected to express full-length merlin, those that do are likely to possess informative missense mutations and such mutations would be invaluable for defining functional residues involved in critical protein-protein interactions.

The papers that have resulted from this work (covered under this co-operative agreement) are listed below. Papers are enclosed in the appendix.

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Louis D, Ramesh V, Gusella J. Neuropathology and Molecular Genetics of Neurofibromatosis 2 and related tumors. Brain Pathology, 1995; 5:163-172.

Gusella J, Ramesh V, MacCollin M, Jacoby L. Neurofibromatosis 2: loss of merlin's protective spell. Current Opinion in Genetics and Development 1996; 6:87-92.

Presentation at Meetings

Nf2 gene structure and mutational analysis, American Society of Human Genetics, October 1993.

Neurofibromatosis 2, Faseb conference on Neurofibromatosis, July 1994.

- *Cellular localization of the NF2 protein merlin, The National Neurofibromatosis Foundation meeting, July 1995*
- Expression and localization of epitope-tagged merlin, The National Neurofibromatosis Foundation meeting, July 1995.*
- Cellular localization of endogenous and epitope-tagged Nf2 protein merlin, American Society of Human Genetics, October 1995.*
- Expression and cellular localization of the Nf2 protein merlin, FASEB conference on Neurofibromatosis, July 1996.*
- The NF2 tumor suppressor protein merlin localizes preferentially in membrane ruffles, Cytoskeletal regulation of membrane function, September 1996.*

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APPENDIX

Mutations in transcript isoforms of the neurofibromatosis 2 gene in multiple human tumour types

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The neurofibromatosis 2 gene (*NF2*) has recently been isolated and predicted to encode a novel protein related to the moesin-ezrin-radixin family of cytoskeleton-associated proteins. Here we describe a novel isoform of the *NF2* transcript that shows differential tissue expression and encodes a modified C terminus of the predicted protein. Mutations affecting both isoforms of the *NF2* transcript were detected in multiple tumour types including melanoma and breast carcinoma. These findings provide evidence that alterations in the *NF2* transcript occur not only in the hereditary brain neoplasms typically associated with *NF2*, but also as somatic mutations in their sporadic counterparts and in seemingly unrelated tumour types. The *NF2* gene may thus constitute a tumour suppressor gene of more general importance in tumorigenesis.

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Neurofibromatosis type 2 (*NF2*) is an autosomal dominantly inherited condition whose hallmark is the development of bilateral vestibular schwannomas (acoustic neuromas) of the eighth cranial nerve. Other disease features include cranial meningiomas, spinal nerve root schwannomas and presenile lens opacities¹⁻³. On the basis of genetic linkage studies and analysis of deletions in sporadic and familial tumours, it has been proposed that loss or inactivation of a tumour suppressor gene in the *NF2* locus in chromosome 22q leads to tumorigenesis⁴⁻¹¹. Physical mapping and positional cloning studies have culminated recently in the identification of a candidate *NF2* gene^{12,13}. Recently, we demonstrated nonoverlapping deletions affecting the candidate *NF2* gene region in DNA from three independent *NF2* families and in mRNA from a meningioma in an unrelated *NF2* patient¹². Germ-line and somatic mutations have been also demonstrated in the candidate *NF2* gene in DNA from both *NF2* patients and *NF2*-related tumours, including sporadic meningiomas and vestibular schwannomas¹³. The *NF2* gene encodes a 595 amino acid protein, called *merlin* (for moesin-ezrin-radixin like protein) that exhibits significant homology to a highly conserved family of proteins that have been postulated to connect the cytoskeleton to components of the plasma membrane¹²⁻¹⁴ (the corrected *NF2* gene cDNA sequence is under accession number L11353). Cloning of a candidate *NF2* gene was independently confirmed by Rouleau *et al.*, who have designated the *NF2* gene product *schwannomin*¹⁵. Among the family members, *merlin* shows the most extensive homology (63%) to moesin, ezrin and radixin within a region that spans approximately 340 residues at the N terminus of the predicted protein^{12,13}.

The *NF2* gene was found to be expressed in multiple tissues, suggesting that alterations in this gene might be involved in multiple tumour types in addition to the brain neoplasms typically associated with *NF2*. In this regard, cytogenetic and molecular studies have implicated losses in chromosome 22q in several human neoplasms¹⁵, including breast and colon carcinomas, glioblastomas, meningiomas, pheochromocytomas and schwannomas.

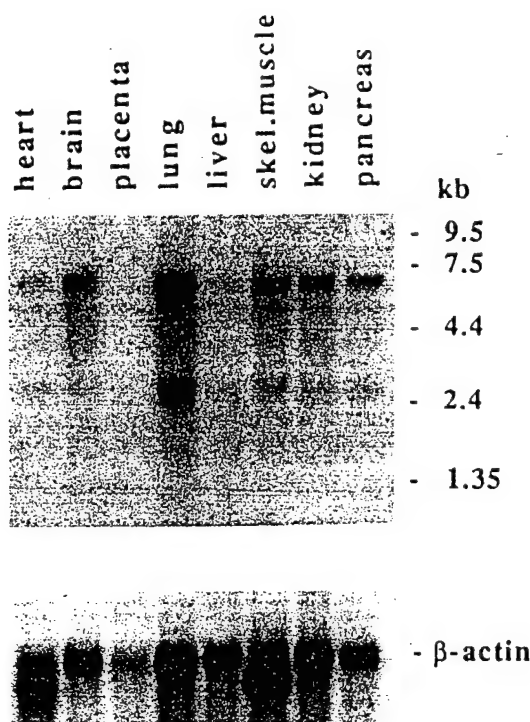
Here we describe RNA polymerase chain reaction (PCR) and single strand conformational polymorphism (SSCP) analyses designed to survey different tumour types for mutations within the coding region of the *NF2* mRNA. In addition to the *NF2* gene alterations detected so far in hereditary and sporadic vestibular schwannomas and meningiomas^{12,13}, our results also demonstrate the presence of *NF2* gene transcript mutations in neoplasms seemingly unrelated to *NF2*, including breast carcinoma and malignant melanoma.

Moreover, we identified a novel isoform of the *NF2* gene transcript generated by a 45 basepair (bp) insertion that encodes eleven amino acids and a premature termination codon, thus resulting in a variable C terminus of the predicted *NF2* protein. The presence of an alternative splice site and the occurrence of deletions affecting the C terminus of the predicted protein suggest an important role for this region in tumour suppressor function.

Thus, mutations in *NF2* are not restricted to central nervous system (CNS) tumours, suggesting a more general involvement of this gene in cancer development.

Expression of the *NF2* gene in human tissues

To evaluate the pattern of expression of the *NF2* gene, we performed northern blot analysis using poly(A)⁺ RNA



isolated from various human tissues. Two major transcripts of approximately 2.6 and 7 kilobases (kb), together with a weakly hybridizing species of 4.4 kb, were detected in heart, brain, lung, liver, skeletal muscle, kidney, pancreas, and placenta (only a very faint signal was observed in overexposed Northern blots, for the latter) (Fig. 1). PCR analysis also revealed the presence of NF2 transcripts in total RNA extracted from the eighth cranial nerve, adrenal

Fig. 1 Northern analysis of polyA⁺ RNA from different human tissues. Transcripts of approximately 7 kb, 4.4 kb (very weak signal) and 2.6 kb can be detected in most tissues by hybridization with a full-length NF2 cDNA probe. A very faint signal was detected in placenta in overexposed films. A beta-actin control for RNA integrity is shown at the bottom.

gland and cerebellum (see below), indicating that expression of NF2 is not restricted to the CNS.

Analysis of vestibular schwannomas

To investigate the presence of mutations within the NF2 coding region in tumours typically associated with NF2, a PCR/SSCP analysis was performed in RNA from vestibular schwannomas.

A total of twelve sporadic and three NF2-associated vestibular schwannomas were screened (Table 1). A preliminary analysis of the PCR-amplified products by agarose gel electrophoresis revealed fragments of reduced size in five tumour samples, including tumours AN10 and AN825 (Fig. 2a and c). SSCP analysis of the remaining cases detected a variant electrophoretic pattern in three additional tumours, including AN13 (Fig. 2b). Cloning and double strand sequencing of the aberrant RNA PCR fragments demonstrated the presence of mutations in each of the tumours showing products of altered size. As summarized in Table 1, mutations within the NF2 coding region were detected in two of three hereditary and in six of twelve sporadic vestibular schwannomas. One tumour (AN11) carried an NF2 cDNA deletion that introduced a frameshift, resulting in premature termination of the reading frame within 56 bp of the deletion breakpoint (Table 1). The remaining seven vestibular schwannomas exhibited in-frame transcript deletions that would result

in truncated proteins without altering the distal reading frame (Table 1). In some tumours (AN94, AN10, AN54, AN72 and AN825), in-frame cDNA deletions represented whole exons. Some of these transcript deletions could potentially involve mutations in splice junctions, but this has not been confirmed due to the lack of DNA from these tumours. The total absence of RNA PCR products derived from the normal allele was observed in five vestibular schwannomas (Table 1, tumours AN94, AN11, AN13, AN825 and AN72), consistent with the highly homogeneous nature of this tumour type which only exhibits minor contamination of non-neoplastic tissue.

The origin of the mutations was confirmed by screening lymphocyte DNA from the patients. Mutation analysis of blood DNA from the patients bearing sporadic vestibular schwannomas AN94, AN10, AN11, AN54, AN72 and AN825 did not reveal any alterations in the NF2 gene, including the exon/intron junctions

Table 1 NF2 gene transcript mutations in human tumours

Tumour ^a	Histopathology ^b	Mutation ^c	Position ^d	Effect ^e
1) AN94 (s)	VS (S)	Δ135 bp	676-810	Δ (I)
2) AN10 (s)	VS (S)	Δ84 bp	364-447	Δ (I)
3) AN11 (s)	VS (S)	Δ163 bp	1575-1737	(f) > stop(1793)
4) AN54 (s)	VS (S)	Δ126 bp	115-240(113-238)	Δ (I)
5) AN72 (s)	VS (S)	Δ84 bp	364-447	Δ (I)
6) AN825 (s)	VS (S)	Δ114 bp	886-999(888-1001)	Δ (I)
7) AN13 (h)	VS (N/A)	Δ18 bp	358-375	Δ (I)
8) AN26 (h)	VS (N/A)	Δ57 bp	433-489	Δ (I)
9) 86336	breast ductal CA	Δ211 bp	600-810	(f) > stop(884)
		A > T	817	Ile273 > Phe
10) 94771	melanoma	D87 bp	1000-1086 (1003-1089)	Δ (I)
11) 95540	melanoma (m)	Δ85 bp	361-445	(f) > stop(518)
12) 87506	melanoma (m)	Δ228 bp	1504-1731 (1501-1728)	Δ (I)
		A > T	1091	Lys364 > Ile
13) 95783	melanoma (m)	Δ125 bp	1447-1571	(f) > stop(1646)
14) 86-20	melanoma (m)	Δ143 bp	1123-1265	(f) > stop(1323)
15) 90021	melanoma (m)	Δ1 bp	616	(f) > stop(623)

^as, Sporadic; h, hereditary tumour.

^bVS, Vestibular schwannoma; S, somatic origin of the mutation; N/A, no blood from the patient was available; CA, sarcoma; m, metastasis.

^cΔ, Deletion. The number of base pairs deleted follows the corresponding symbol.

^dNucleotide position of the mutations relative to the NF2 gene initiation codon. Alternative nucleotide positions (due to the repeated nature of the deleted sequence boundaries) are given in parenthesis.

^eD, Deletion; I, in-frame; f, frameshift. The number in parenthesis indicates the nucleotide position of the stop codon generated by the frameshift.

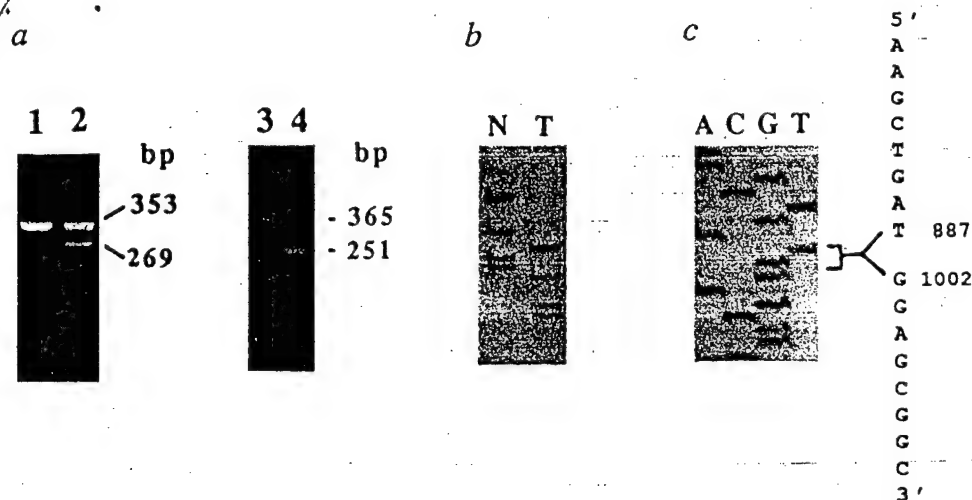


Fig. 2 *NF2* transcript deletions in vestibular schwannomas. **a**, RNA-PCR products electrophoresed in a 3% agarose gel and stained with ethidium bromide. Lanes 1 and 3, total RNA from the eighth cranial nerve using primer sets 5m2-3m2 and 5m4-3m4, respectively. Lanes 2 and 4, vestibular schwannomas AN10 and AN825 showing deletions in the *NF2* transcript as evidenced by the presence of fragments of 269 bp and 251 bp, respectively. **b**, Abnormal conformer detected by SSCP analysis in RNA from vestibular schwannoma AN13 (T) using primers 5m2-3m2. Lane N represents the SSCP conformer from the eighth cranial nerve. **c**, DNA sequence showing a deletion of 114 bp between nucleotides 887 and 1002 in vestibular schwannoma AN825.

flanking the sequences deleted in the tumour cDNAs, thus confirming the somatic nature of these mutations (Table 1).

Analysis of tumours seemingly unrelated to *NF2*

Since losses affecting chromosome 22q have been reported in a variety of neoplasms¹⁵, we reasoned that somatic mutations in the *NF2* gene might be implicated not only in tumours typically associated with *NF2* but also in seemingly unrelated cancers. Therefore, different tumour types from non-*NF2* individuals, including breast and colon carcinomas, malignant melanomas and pheochromocytomas, were screened for mutations within *NF2* coding region.

Because *NF2* is predominantly associated with tumours derived from the embryonic neural crest, we first analysed a series of sporadic pheochromocytomas and melanomas, two tumour types that do not show a higher incidence in *NF2* patients. Of three primary melanomas and seventeen melanoma metastases analysed, sequencing of aberrant SSCP conformers demonstrated the presence of *NF2* gene transcript mutations in six tumours. In-frame deletions

were detected in one primary skin melanoma (tumour 94771; superficial spreading type) and one melanoma metastasis (tumour 87506); the latter also exhibited an A to T transversion resulting in a nonconservative substitution of lysine to isoleucine (Table 1). The mutations detected in the remaining four melanoma metastases consisted of deletions of 1–143 bp that altered the reading frame generating premature stop codons (Table 1). A restriction enzyme map of the *NF2* transcript indicated that the deletion observed in tumour 95540 would result in loss of a *Bgl*II site. Southern blot analysis of tumour DNA digested with *Bgl*II revealed an aberrant restriction pattern as compared to the control DNA (data not shown), suggesting that the cDNA deletion described above is present at the genomic level. No apparent mutations in the *NF2* cDNA were detected in five sporadic pheochromocytomas screened by RNA PCR/SSCP analysis.

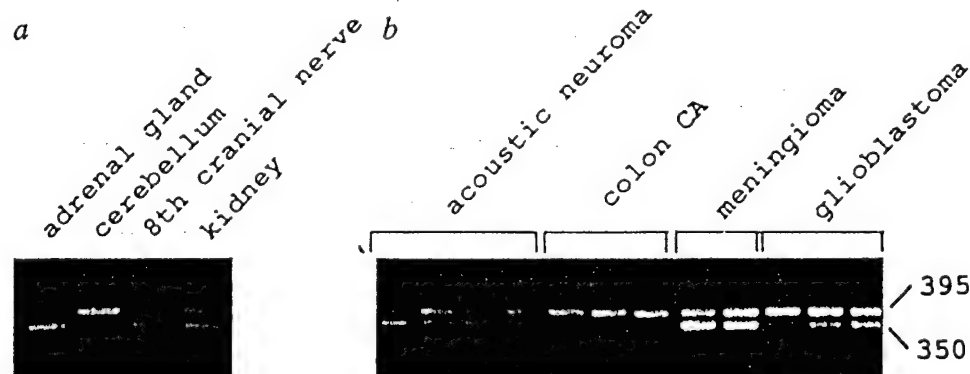
We further investigated the presence of *NF2* transcript mutations in breast and colon carcinomas. Of fourteen primary breast carcinomas analysed by RNA PCR/SSCP methods, one tumour showed a fragment of altered mobility. Sequence analysis of the altered-size PCR product demonstrated that this tumour, diagnosed as a poorly differentiated breast ductal carcinoma, carried a deletion of 211 bp that resulted in a frameshift, thereby introducing a premature termination codon within 74 bases of the deletion breakpoint (Table 1). Sequencing of this product also showed an A to T transversion at nucleotide 817 that would result in a substitution of isoleucine by phenylalanine (Table 1). This mutation was not detected in DNA from normal surrounding tissue, thus ruling out the possibility of a polymorphism (not shown).

No apparent mutations in the *NF2* coding region were detected in RNA samples extracted from twenty colon carcinomas.

Alternative splicing of the *NF2* transcript

PCR analysis of the *NF2* gene transcript using primers 5m6 and 3m6 revealed the presence of two distinct

Fig. 3 Detection of two isoforms of *NF2* transcripts in different tissues and tumour types by RNA-PCR analysis. **a**, Ethidium bromide-stained electrophoresis pattern of co-amplified products derived from isoform I (350 bp) and isoform II (395 bp) *NF2* transcripts in four human tissues. **b**, Pattern of isoform I and isoform II *NF2* gene transcripts in different tumour types.

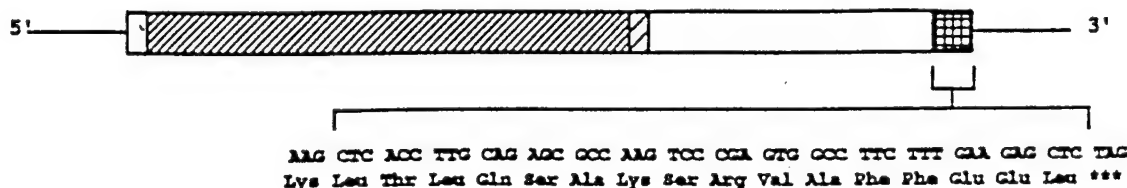


products of 350 bp and 395 bp in different tissues including the eighth cranial nerve (the tissue from which vestibular schwannomas derive) (Fig. 3). Analysis of one of these transcripts confirmed a sequence identical to the one reported previously^{12,13}, whereas the product with higher molecular weight revealed a 45 bp insertion at nucleotide 1737, encoding eleven amino acids and a premature

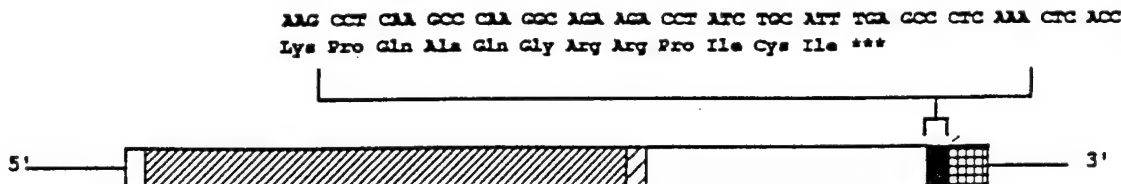
termination of the reading frame (Fig. 4a). This insertion was also detected in an *NF2* cDNA clone isolated from a fetal brain cDNA library (data not shown). The novel isoform of the *NF2* transcript, which presumably arises by alternative splicing, encodes a 590-amino acid protein with a modified C terminus. Therefore, the predicted C terminus of both *NF2* proteins would differ by a total of

a

NF2 gene transcript isoform I

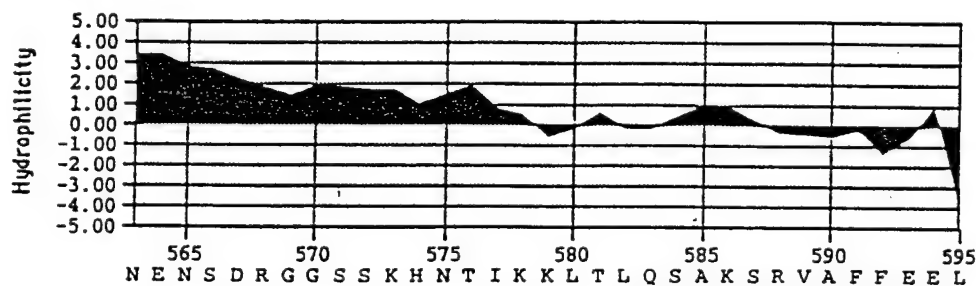


NF2 gene transcript isoform II



b

Merlin I



Merlin II

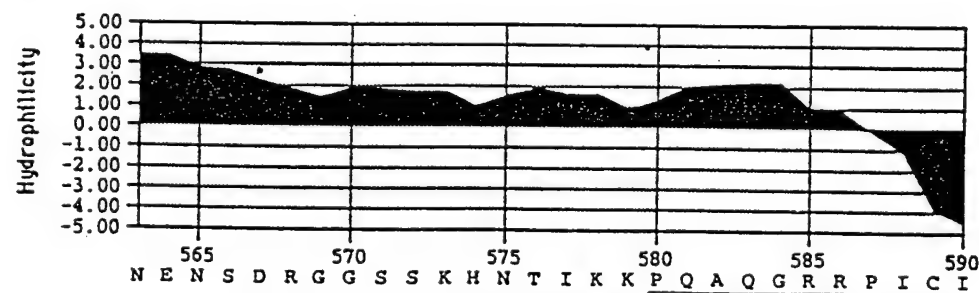


Fig. 4 *NF2* gene transcript isoforms. a, Partial nucleotide and predicted amino acid sequence at the 3' end of isoforms I and II of the *NF2* transcript. The checkered box in isoform I indicates the nucleotide sequence encoding a variable C terminus of 16 amino acids. The black box indicates the 45 bp insertion, and the predicted amino acid sequence that gives rise to isoform II. Coding regions with high (densely hatched box) and partial (hatched box) homology to moesin, ezrin and radixin, are shown. b, Comparison of hydropathic patterns between isoforms I and II of the predicted merlin protein. Hydropathy of the regions from Asn563 to Leu595 of isoform I and from Asn563 to Ile590 of isoform II was analyzed by Kyte-Doolittle¹⁶ plotting. The variable regions at the predicted C terminus of isoforms I and II of *merlin* are underlined.

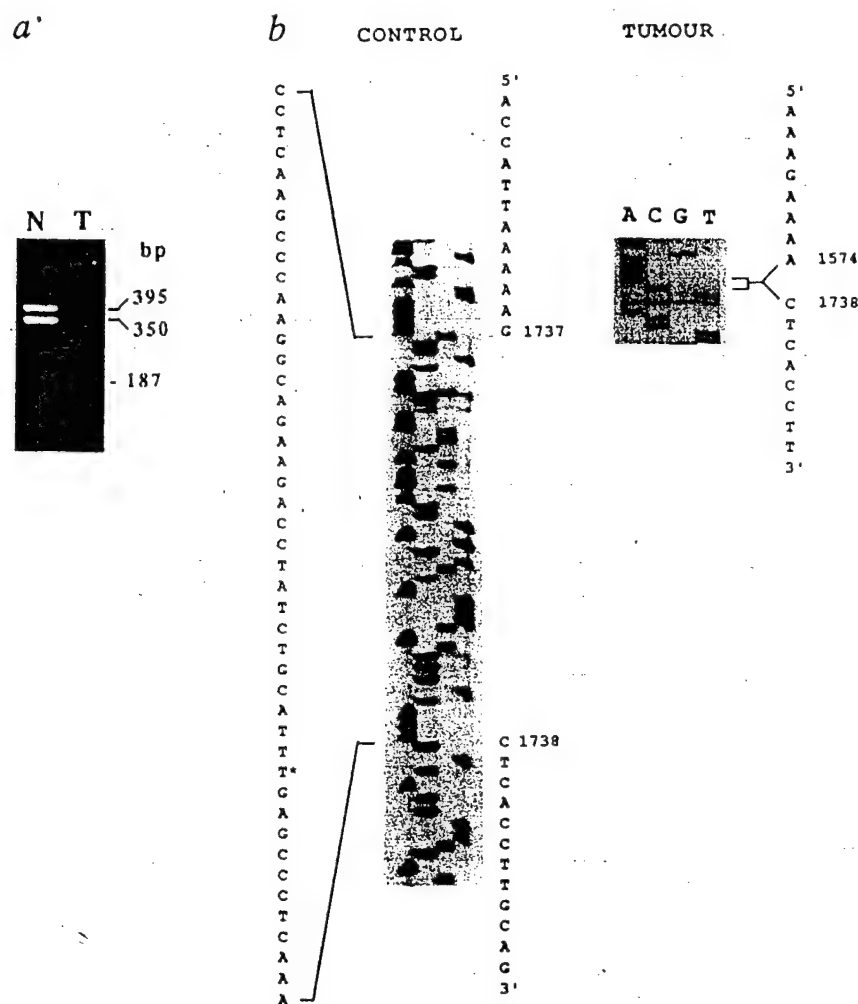


Fig. 5 Deletion at the predicted C terminus of two *NF2* transcript isoforms. *a*, The presence of two distinct RNA PCR products (395 bp and 350 bp) was detected in normal tissue (N) using primers 5m6 and 3m6. Vestibular schwannoma AN11 (T) shows a unique fragment of reduced size (187 bp). *b*, Partial nucleotide sequence of the 395 bp RNA PCR product from normal tissue (control), showing the 45-bp insert present in isoform II of the *NF2* transcript. The novel termination codon is marked by an asterisk. A partial sequence of the RNA PCR product from vestibular schwannoma AN11 (tumour) demonstrates the presence of a deletion (nucleotides 1575-1737) that includes the alternative splice site at nucleotide 1737.

sixteen amino acids (Fig. 4a). We have designated the novel *NF2* splice variant as isoform II, and the original *NF2* transcript^{12,13} as isoform I.

Secondary structure analysis and hydrophobicity plotting¹⁶ revealed marked differences between the predicted C-terminal regions of isoforms I and II of *merlin* (Fig. 4b). The novel eleven amino acid peptide sequence of isoform II starting at proline 580 gives rise to a non-helical more hydrophilic C terminus. By contrast, the C-terminal amino acid sequence of the predicted isoform I is more hydrophobic and exhibits a helical structure (Fig. 4b).

The location and predicted amino acid sequence of the isoform II splice variant are completely conserved in the mouse (data not shown). RNA PCR analysis also shows marked differences in the relative abundances of both *NF2* transcripts in a number of tissues and tumour types (Fig. 3). Whereas isoform II shows very low relative expression levels in adrenal gland and the eighth cranial nerve, isoform II is the predominant species in RNA from

cerebellum (Fig. 3a). By contrast, both isoforms have similar expression levels in kidney (Fig. 3a). Similarly, differences in the relative expression levels of isoform I and II transcripts are found in different tumour types (Fig. 3b). In particular, isoform II was expressed predominantly in three colon carcinomas analyzed with almost complete absence of isoform I (Fig. 3b).

RNA PCR analysis of one vestibular schwannoma (AN11) using oligonucleotide primers that flank the alternative splice site near the 3' end of the *NF2* gene transcript (5m6 and 3m6) showed a unique fragment of reduced size (187 bp) in comparison with the products derived from isoforms I and II (350 bp and 395 bp, respectively) in normal tissue (Fig. 5a). Sequencing of the aberrant product confirmed a 163 bp deletion that included the alternative splice site at nucleotide 1737 (Table 1; Fig. 5b). This deletion near the 3' end of the *NF2* transcript would result in the removal of 54 amino acids, including the C-terminal end of isoform II, and introduce a frameshift in the reading frame of isoform I.

Discussion

NF2 mutations in multiple tumour types. We have previously identified a candidate *NF2* gene based on the presence of nonoverlapping constitutional deletions in DNA from three unrelated *NF2* families and the detection of aberrations in mRNA in a meningioma from an independent *NF2* patient¹². Here, the detection of homozygous or hemizygous deletions of somatic origin within the candidate *NF2* coding region in sporadic

tumours (AN11, AN94, AN72 and AN825) provides clear evidence for the specific inactivation of both *NF2* copies and strongly suggests that *NF2* acts as a recessive tumour suppressor in accordance with the two-hit model of tumorigenesis¹⁷.

In addition to the hereditary brain tumours commonly found in *NF2* patients, our results confirm that *NF2* mutations may also contribute to the tumorigenesis of sporadic schwannomas. Deletions and point mutations in *NF2* were previously reported in two of 30 sporadic vestibular schwannomas and two of 30 sporadic meningiomas¹³. Here, *NF2* transcript mutations were detected in six of 12 sporadic vestibular schwannomas. Similarly, a separate analysis of sporadic meningiomas (manuscript in preparation) shows a higher frequency of *NF2* alterations than previously shown¹³. Thus our results confirm that *NF2* appears to play a role in the tumorigenesis of sporadic schwannomas and meningiomas, the relative incidence of which is significantly higher than their hereditary (*NF2*-related) counterparts, accounting for

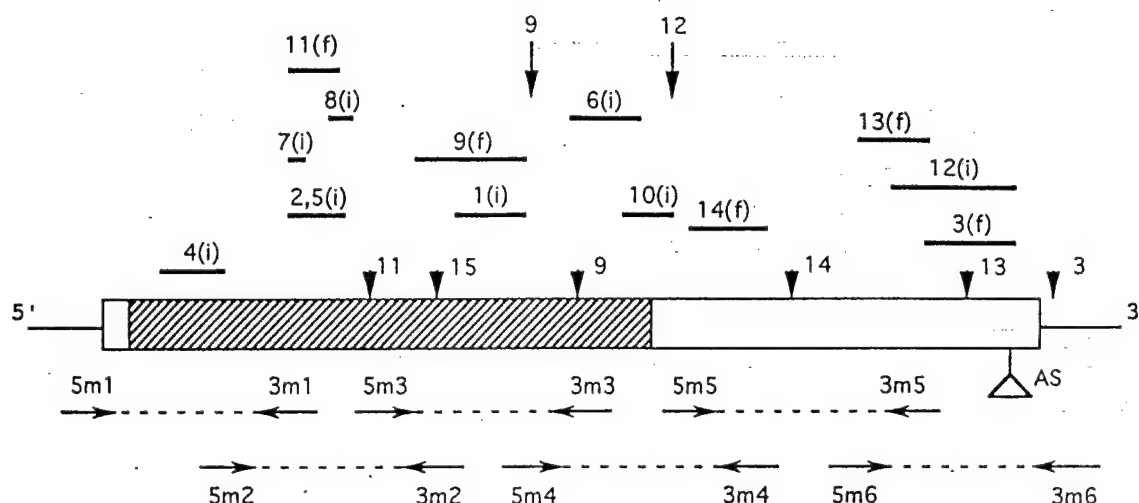


Fig. 6 Mutations in the *NF2* transcript in human tumours. Schematic drawing showing the relative position of *NF2* mutations in different human tumour types. Numbers represent the tumours listed in Table 1 (1-15). Solid lines represent in-frame (i) or frameshift (f) deletions. Vertical arrows indicate point mutations. The vertical arrow for sample no. 15 (melanoma 90021) represents a 1 bp deletion. The location of stop codons generated by frameshifts in the reading frame is indicated by solid vertical triangles. The coding region homologous to *moesin*, *ezrin* and *radixin* is represented by a hatched box. AS, alternative splice site. PCR oligonucleotide primers are represented by horizontal arrows.

approximately 21–26% of all intracranial neoplasms in adults¹⁸.

In addition to the CNS neoplasms typically associated with *NF2*, mutations in *NF2* appear to be implicated in seemingly unrelated tumour types. In this regard, the detection of mutations within the *NF2* coding region in six malignant melanomas, a tumour type not known to be more frequent in *NF2* patients, suggests that inactivation of *NF2* may represent a significant step in the development of this malignancy. In addition to brain tumours and melanomas, which are both derived from tissues of neural crest origin, the detection of *NF2* mutations in a breast carcinoma suggests that this gene might also be implicated in neoplasms derived from tissues of different embryological origin. Different frequencies of allelic losses of chromosome 22q have been previously reported in breast carcinomas^{19–21}. Interestingly, one study showed total or partial loss of chromosome 22 in six of eight lobular breast carcinomas as opposed to two of 28 ductal breast tumours¹⁹. Although the latter frequency is consistent with our findings, a comprehensive analysis of breast tumours of different histopathological types will be necessary to assess the actual percentage of *NF2* alterations in breast cancer. Similarly, further screening of a larger set of tumours will be required to determine whether *NF2* mutations are implicated in other malignancies where chromosome 22 allelic losses have been reported, including pheochromocytomas²², gliomas²³ and colon carcinomas^{24,25}.

The finding of *NF2* mutations in sporadic tumours not typically associated with *NF2* is reminiscent of other hereditary cancer syndromes, including familial retinoblastoma, Li Fraumeni's syndrome and *NF1*. Tumour suppressor genes associated with these disorders have also been implicated in sporadic malignancies that do not occur more frequently in patients with germline mutations^{26–29}. *NF2* mutations may therefore be rate-limiting^{17–30} in tumour types associated with *NF2* but represent later stages in the

tumour progression of *NF2*-unrelated neoplasms.

An initial understanding of the functional significance of the domains of the merlin protein may be gained from the location of the *NF2* deletions reported here and their predicted effects on protein structure. Interestingly, most of the alterations (15 of 17 mutations, Fig. 6) are predicted to generate truncated proteins as the result of in-frame deletions or premature stop codons. In most cases, these truncations would result in the removal of distal domains, including the α -helical and C-terminal regions, from the merlin protein. Although in some tumours the reading frame is preserved distal to the deletion breakpoint, it is likely that these dramatic alterations, which in some cases comprise whole exons, disrupt domains critical for merlin protein function. Evidence for function of the C terminus of merlin is provided by the frameshift deletion detected in vestibular schwannoma AN11 (Table 1; Fig. 5), representing the most distal alteration described here. In this regard, the homozygous nature of this mutation, which predicts the removal of 54 C-terminal amino acids, suggests that this region might be of critical importance for the putative tumour suppressor function of merlin.

We have also identified two point mutations that result in nonconservative changes in codons 273 and 364 (Table 1). Similarly, a nonconservative Leu360Pro substitution that segregates with *NF2* was previously reported¹³. Further studies should provide information on the functional significance of these substitutions. Overall, our results suggest that mutations leading to a truncated protein constitute the predominant mechanism for the inactivation of the *NF2* product. These findings are in agreement with previous reports^{12,13} in which the majority of alterations detected in hereditary tumours predicted a truncated protein.

Expression of a novel *NF2* transcript isoform. The functional importance of the C-terminal region in merlin is also suggested by the location and amino acid sequence conservation (mouse versus human) of a putative

alternative splice variant leading to a predicted protein isoform (designated isoform II) with a C terminus differing from isoform I by 16 amino acids. In this context, the marked differences in hydrophilicity and secondary structure of these C-terminal regions suggest that this region may represent a functionally distinct domain whose variability may result in products with modified cellular function. Alternative splicing is a frequent mechanism for generating multiple isoforms resulting in functionally different products. In particular, heterogeneity due to differential splicing has been shown to result in modified sites of interaction with other proteins or physiological targets³¹. In this regard, it has recently been suggested that alternative splicing in the C-terminal region of erythrocyte protein 4.1, a member of the moesin family, may be critical for its binding to spectrin and for the mechanical integrity of the red cell membrane³². The marked differences observed in the relative abundance of isoforms I and II in various tissues may therefore reflect a degree of functional complexity in the merlin protein.

The possibility that mutations in distinct domains and isoforms of merlin could be associated with different phenotypes is particularly interesting in view of the various clinical features related to NF2. In this regard, two clinical forms of NF2 have been described³. The characterization of additional mutations may thus allow us to establish more precise pathogenetic associations.

In conclusion, we provide evidence for the involvement of NF2 alterations in multiple tumour types. The availability of cloned truncated mutant forms of merlin and of normally occurring alternative splice variants will provide useful tools for the analysis of the normal function of the merlin protein. Future studies should facilitate new insights into the role that interactions between the cytoskeleton and plasma membrane play in tumorigenesis.

Methodology

Northern analysis. A northern blot (Clontech Laboratories) containing 2 µg of poly A⁺ RNA from eight human tissues was hybridized to a [α -³²P] dCTP-labelled probe generated by reverse transcription/PCR amplification of the entire NF2 coding region. Filter hybridization was performed at 68 °C for 3 h using the QuickhybTM solution (Stratagene), followed by two washes with 2 × SSC/0.1% SDS for 15 min at room temperature, and one wash with 0.1 × SSC/0.1% SDS for 15 min at 54 °C.

RNA PCR amplification. Total RNA was extracted from frozen tumour specimens by lysis in guanidium thiocyanate and extraction with phenol-chloroform³³. Total RNA was denatured by heating to 70 °C for 10 min in 13 µl of DEPC-treated water. After chilling on ice for 2 min, single-stranded cDNA was synthesized by incubating the denatured RNA in 20 µl of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM MgCl₂, 100 ng µl⁻¹ BSA, 500 µM dNTP, 10 mM DTT, 12 ng µl⁻¹ of primer 3m3 or 3m6 and 200 U of SuperScriptTM (Gibco BRL) reverse transcriptase for 10 min at room temperature followed by 60 min at 42 °C. The reaction was terminated by heating to 95 °C for 2 min and quenching on ice. A first amplification by PCR was performed using 2 µl of the reverse-transcribed product in a final volume of 100 µl of 20 mM Tris-HCl (pH 8.2), 10 mM KCl, 6 mM (NH₄)₂SO₄, 1.5 mM MgCl₂, 0.1% Triton X-100, 2.5 U Pfu DNA polymerase (Stratagene), 200 µM each deoxynucleoside triphosphate and 0.3 pmole µl⁻¹ of primers 5m1 and 3m3 (for the 3m3-primed first strand cDNA) or 5m4 and 3m6 (for the 3m6-primed product). Nested PCR amplifications were performed using 1 µl of first

amplification product in a final volume of 100 µl of solution containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.001% (w/v) gelatin, 200 µM each deoxynucleoside triphosphate, 0.3 pmole µl⁻¹ of appropriate primers and 2.5 U Taq polymerase (Boehringer Mannheim). 30 cycles of amplification were performed in a GeneAmp 9600 machine (Perkin Elmer), with denaturation at 94 °C for 15 s, annealing at 58 °C for 15 s and elongation at 72 °C for 1 min 15 s. The oligonucleotide primers used in reverse transcription and PCR amplification were as follows: 5m1: 5'-CATGGCCGGGCCATCGCTTCC-3'; 3m1: 5'-CCTGA-ACCAGCTCCTCTTCAGC-3'; 5m2: 5'-TCAAAGGAAGAACC-AGTACC-3'; 3m2: 5'-TCAGCTTCATCCCTGGCTCG-3'; 5m3: 5'-GGAGAGAATTACTGCTTGGTAC-3'; 3m3: 5'-CATAAA-TAGATCATGGTTCCCGAT-3'; 5m4: 5'-CCTCAAAGCTT-CGTGTTAATAAGC-3'; 3m4: 5'-TTCCTGCTCAGCCTCTGCGGC-3'; 5m5: 5'-GGAGGCAAACTTCTGGCCAG-3'; 3m5: 5'-GACAGGCTGTACCAATGAGG-3'; 5m6: 5'-CAATT-CCAGCACCGTTGCCCTCC-3'; 3m6: 5'-GGGTGGCTGGGTG-ACCTGCT-3'. Tumour samples listed in Table 1 were amplified by RNA PCR using the following oligonucleotide primer sets: AN54: 5m1-3m1; AN10, AN13, AN72, AN26, 95540: 5m2-3m2; AN94, 86336, 90021: 5m3-3m3; AN825, 94771: 5m4-3m4; 86-20: 5m4-3m5; AN11, 87506, 95783: 5m5-3m6.

SSCP analysis. Nested PCR amplifications were performed for SSCP analysis using the following reaction mixture (final volume 50 µl): 1 µl of PCR-amplified template, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.001% (w/v) gelatin, 200 µM each dCTP, dGTP, dTTP, 20 µM dATP, 0.1 µl of [α -³²P] dATP (3,000 Ci mmol⁻¹) (Du Pont NEN), 0.3 pmole µl⁻¹ of appropriate primers, and 1.25 U Taq polymerase. Following amplification, PCR products were diluted (1:10) with 0.1% SDS/10 mM EDTA. A 5 µl sample of the diluted reaction was then mixed with 6 µl of gel loading dye (U.S. Biochemical Corporation). Samples were heat denatured at 94 °C for 2 min, chilled on ice, and 3 µl loaded onto a 0.5 × MDE gel (J.T. Baker). Gels were electrophoresed at 8 Watts constant power for 14 h at room temperature using 0.6 × TBE buffer. After electrophoresis, gels were transferred to blotting paper, dried and subjected to autoradiography.

Genomic DNA PCR analysis. Genomic DNA extracted from the patients' blood was analysed by PCR using oligonucleotide primers that flank the exon-intron junctions of the NF2 gene. Primer sequences are described elsewhere (Jacoby *et al.*, manuscript in preparation).

Sequence analysis. Individual bands were carefully excised from agarose gels or from dried SSCP gels, placed into 100 µl of deionized water, and the DNA allowed to elute for 4–6 h at room temperature with gentle shaking. 10 µl of eluted DNA was reamplified using appropriate primers as described above. Amplified products were subcloned into the plasmid vector pCRTM II (Invitrogen), and inserts were sequenced using double-stranded recombinant plasmids as template for the dideoxy chain termination method³⁴. Reaction products were electrophoresed on 6% polyacrylamide, 8 M urea, 0.1 M Tris-borate (pH 8.3), 2 mM EDTA gels. Some of the clones were sequenced by dideoxy terminator chemistry using an Applied Biosystems 373-A automated DNA sequencer. Double strand sequencing of three clones was performed for each mutant sample. Sequence data was analyzed using the MacVectorTM 4.1 software (International Biotechnologies).

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The murine *NF2* homologue encodes a highly conserved *merlin* protein with alternative forms

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The recently isolated gene for neurofibromatosis type 2 (*NF2*) encodes a 595 amino acid protein, named *merlin*, which is related to the cytoskeleton-associated proteins moesin, ezrin and radixin. To identify evolutionarily conserved regions and to provide sequence information necessary for the establishment of a mouse model for *NF2*, we have determined the cDNA sequence of the mouse *NF2* tumor suppressor gene, and mapped it in the mouse genome. Mouse *merlin* is a 596 amino acid protein, 98% identical to human *merlin*, but one amino acid longer due to the insertion of a proline residue near the C-terminus. Of the nine amino acid differences between mouse and humans, seven occur in the C-terminal 20% of the protein, far from the protein 4.1 domain that defines this family. Two of the *NF2* cDNA clones reveal evidence of alternative splicing events that alter the predicted *merlin* product, one removing a 45 amino acid segment from the middle section of the protein and the other changing the C-terminus. The existence of several different forms of *merlin* potentially with different primary roles will complicate the identification of the precise function that must be disrupted to cause the *NF2*-associated tumors. The mouse *NF2* homologue maps to Chr 11, in a region homologous to human Chr 22, but devoid of any mouse mutations which could be models of the human disorder.

INTRODUCTION

Neurofibromatosis 2 (*NF2*), previously known as central neurofibromatosis, is a severe inherited disorder that is genetically distinct from the more common and widely recognized neurofibromatosis 1, or von Recklinghausen neurofibromatosis (1,2). Both disorders involve the disordered growth of Schwann cells, but *NF2* is characterized by the formation of benign tumors affecting mainly the central nervous system. The hallmark of *NF2* is the bilateral occurrence of vestibular schwannomas on the eighth cranial nerve, but meningiomas, ependymomas and schwannomas of other cranial nerves and of spinal nerve roots are also frequent. Some patients also display posterior capsular opacities (3). Symptoms typically begin in the teenage years or

early adulthood as slow growing tumors that gradually cause deafness, loss of balance control, paralysis and early death.

NF2 has an incidence of approximately 1 in 40,000 (4) and is caused by a highly penetrant defect in gene that maps to chromosome 22 (5–8). Family studies and tumor analyses have indicated that *NF2* is caused by inactivation of a tumor suppressor gene (5–17). Although the disorder appears to be transmitted in a dominant fashion, at the cellular level the mechanism is recessive, with inheritance of an inactivated *NF2* allele combining with somatic mutation of the remaining normal allele to initiate tumor formation. We have recently used location cloning techniques to isolate the *NF2* gene, and discovered that it encodes a novel member of a family of cytoskeleton-associated proteins characterized by moesin, ezrin and radixin (18). Subsequently, independent cloning of the same gene was reported by others, confirming the identity of the new protein, which we have named *merlin*, as the *NF2* tumor suppressor (19). To identify evolutionarily conserved regions of the protein, and to lay the foundation for creating an animal model of *NF2*, we have isolated, characterized and genetically mapped the murine *NF2* locus encoding mouse *merlin*.

RESULTS

Isolation of mouse *NF2* cDNAs

To isolate cDNA clones from the mouse *NF2* homologue, we used two probes from the JJR-1 cDNA encoding human *merlin* protein (18). A 1.1 kb *HindIII* fragment containing the 5' portion of the JJR-1 cDNA, and the adjacent ~620 bp *HindIII*–*HindIII* fragment spanning the middle of the coding region were used independently for low stringency screening of a randomly primed C1300 mouse neuroblastoma cDNA library. The clones obtained are shown schematically in Figure 1.

The 3' probe yielded cDNAs NB24, NB32 and NB65. All of these clones encode the C-terminal portion of mouse *merlin* and extend well into the 3' untranslated region (UTR), but none ends in a poly(A) tail. The 3' UTR of all three contains a poly(CT) repetitive sequence which is 4 nt shorter in NB24 than in NB32 and NB65, probably due to reverse transcriptase error. Polymerase chain reaction (PCR) amplification across this dinucleotide repeat did not reveal it to be either heterozygous in C1300 or polymorphic in a variety of inbred strains and in *Mus spretus* (data not shown).

The 5' human *NF2* probe yielded cDNAs NB45 and NB55, which overlap with NB24 and NB65. NB45 with an insert of ~450 bp extends furthest 5' but begins 108 codons short of the

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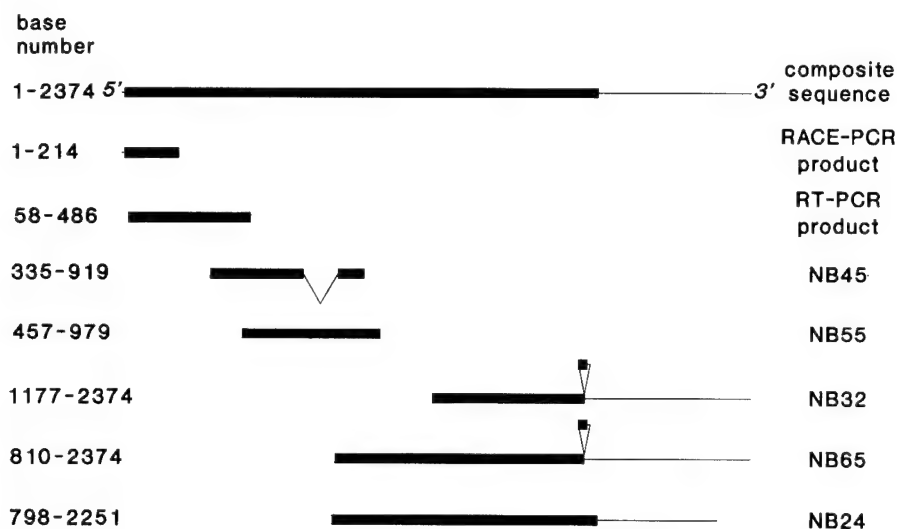


Figure 1. Mouse *NF2* cDNA clones. A schematic diagram is shown of each cDNA clone under the composite map, which ignores alternative splices relative to the human JJR-1 sequence. Coding sequence is shown in the composite map as a thick line while 5' and 3' UTRs are shown as thin lines. The starting and ending bases of each clone are given on the left. Alternative splices are shown relative to the composite map as absence of exon 8 in NB45 and presence of exon 16 in NB32 and NB65.

position predicted by homology with human *merlin* to represent the initiator methionine codon. To isolate most of this missing 5' segment, we reverse transcribed C1300 total RNA with the *NF2* specific primer KM3534 (Figure 2). We then used a primer based on the 5' human sequence, KM3593 and the mouse primer KM3757 to amplify a ~450 bp fragment from the first-strand cDNA. This fragment was directly sequenced. The remaining bases missing relative to the human coding sequence were isolated employing the 5' RACE system (BRL). Several subclones of the amplified fragment were sequenced to eliminate the possibility of Taq polymerase-induced sequence errors.

Mouse and human *merlin* are almost identical in sequence

The composite mouse *NF2* cDNA sequence matching the human JJR-1 clone but omitting sequence added by alternative splicing, is shown in Figure 2. It contains a predicted coding region of 1788 bp, flanked by 10 and 576 bp of 5' and 3' UTR, respectively. Figure 2 also highlights the few DNA and amino acid differences between mouse and human coding sequences. At the DNA level, the mouse and human genes are 90% identical across the coding sequence. The 3' UTRs show 70% identity over the first 100 bp after the stop codon, then diverge, with the mouse sequence extending well beyond the 263 bp 3' UTR of the human JJR-1 clone. Too few bases of the mouse *NF2* 5' UTR were obtained for a meaningful comparison. The murine *merlin* is predicted to contain 596 amino acids, one amino acid larger than human *merlin*, due to the insertion of a proline residue at position 571. There are only nine amino acid differences between mouse and human *merlin*, six of which are conservative changes. Seven of the amino acid differences occur in the C-terminal 20% of the protein, outside of the protein 4.1 family domain in the N-terminal half of *merlin*.

Mouse *NF2* cDNAs are alternatively spliced

Comparison of cDNA NB45 to the composite sequence, as represented by NB55 and to the human JJR-1 clone, showed the

absence of a 135 bp segment ranging from bp 686 to 820 (Figure 2). The missing sequence corresponds precisely with human exon 8 (20) suggesting that at least two *NF2* mRNAs are present in C1300 mouse neuroblastoma cells. This alternative splicing predicts an altered protein product of 551 residues which is missing a 45 amino acid stretch (aa 226–270) from the highly conserved N-terminal half of the protein. To ensure that the two mRNA species indeed result from alternative splicing, rather than from a specific splicing mutation in the C1300 cell line, we performed reverse transcription PCR on total RNA from C1300 neuroblastoma and from adult mouse brain. First strand cDNA was synthesized using two different primers, KM3794 in the 3' UTR and KM4244 in the coding sequence. Each cDNA template was amplified using two different sets of primers, KM3746 and KM4245 which yield products of 811 bp and 676 bp, depending on the presence or absence of exon 8, respectively, and KM3746 and KM3177 which yield products of 549 bp and 414 bp, respectively. In all cases, both with C1300 RNA and with normal mouse brain RNA, two PCR products of the expected sizes were observed (Figure 3). It should be noted that clone NB45 does not contain KM4245, precluding the possibility of contamination in the PCR assay. The relative intensities of the two products suggests that the mRNA missing exon 8 is considerably less abundant than that containing it.

To further complicate the potential variation in the *merlin* protein, sequence analysis of the independent cDNA clones NB32 and NB65 revealed the insertion of a 45 bp segment 16 codons upstream of the *merlin* stop codon. The inserted sequence introduces a new stop codon into the *merlin* reading frame and predicts a slightly shorter protein of 591 amino acids that differs in its last 11 residues from the protein predicted by NB24 and JJR-1. The same sequence, with a single DNA change (Figure 4) has recently been detected as a splice variant (exon 16) in the human *NF2* cDNA (21) and has also been isolated by exon amplification from a human cosmid clone containing the *NF2* gene (Trofatter *et al.*, unpublished).

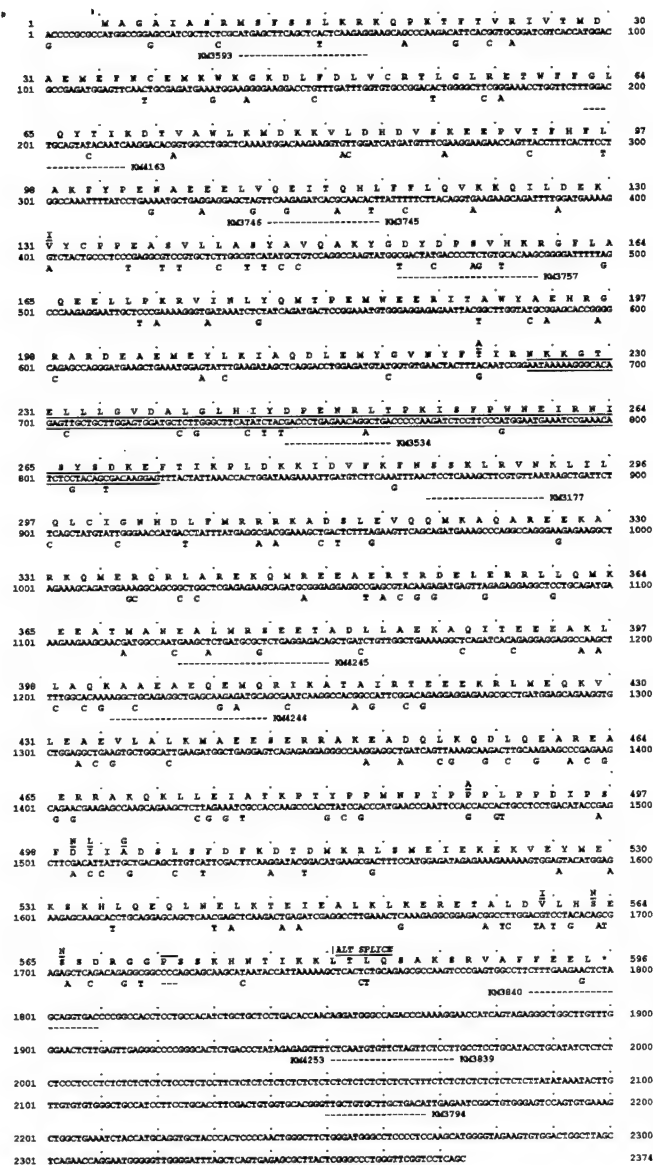


Figure 2. Composite mouse *NF2* cDNA sequence. The composite cDNA sequence of the mouse *NF2* gene is shown, with corresponding amino acids of the mouse *merlin* protein placed above the line. DNA sequence differences relative to the human cDNA JJR-1 are shown below the mouse sequence, only for the coding region. Similarly, amino acid differences relative to human *merlin* are shown above the protein sequence and are underlined. Exon 8 (based on the human exon structure), which is alternatively spliced, is underlined. The site of alternative splicing of exon 16 (based on the human gene structure) is shown by a vertical line. Sequences spanned by PCR primers used in this study are shown below the corresponding DNA sequence, with the 5'-3' orientation of the primer denoted by placing the primer name at the primer's 5' end. The 3' UTR sequence in the vicinity of the poly(CT) stretch was based on NB65 and NB32. NB24 contains one fewer CT pairs in the repeat beginning at bp 2009 and one fewer CT pair in the repeat beginning at bp 2033. The GenBank accession number for this sequence is L27090.

Mouse *merlin* is expressed ubiquitously

Northern blot analysis of human *merlin* expression has revealed three different sized transcripts of 7, 4.4 and 2.6 kb, which are ubiquitously expressed. At least some of this variation is due to alternative polyadenylation (18). A Northern blot survey of mouse

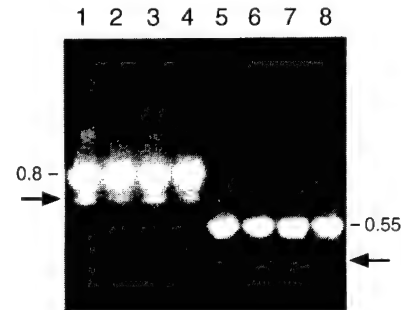


Figure 3. Confirmation of alternative splicing in C1300 and in normal brain. First strand cDNA generated with primers KM3794 (lanes 1, 2, 5 and 6) and KM4244 (lanes 3, 4, 7 and 8) using RNA isolated from normal adult mouse brain (lanes 1, 3, 5 and 7) and from C1300 cells (lanes 2, 4, 6 and 8) was PCR amplified using primer KM3746 along with either KM4245 (lanes 1-4) or KM3177 (lanes 5-8). The approximate size of the major PCR product containing exon 8 is shown. An arrow marks the position of the alternatively spliced product which is missing exon 8. The GenBank accession number of the alternatively spliced form of *merlin* is L27105.

576 N T I K K P Q A Q G R R P I C I * 591
1736 AATACCATTA AAAAGCCTCAAGCCCAAGGCAGAGACCTATCTGCATTG 1785

1786 AGTCCCTCAACTCACTCTGCGAGCGCCAAAGTCCGAGTGGCCTTCTTTG 1835
C CT

Figure 4. Sequence of alternative exon altering the *merlin* C-terminus. The sequence of the alternatively spliced mouse exon 16 is shown underlined, along with sequences flanking its site of insertion from Figure 2. The lone DNA sequence difference relative to the human sequence is shown below the mouse sequence. The predicted protein sequence is shown above the DNA sequence, and shows no difference from that of humans.

merlin expression reveals a single major mRNA size of ~4.5 kb, present in all tissues tested, including heart, brain, lung, liver, skeletal muscle, kidney, and testis (Figure 5). Neither of the alternative splice variants described above would be detectable in the Northern blot assay, leaving open the possibility that either or both might display some tissue specificity. Since no poly(A) tail was found on any of our cDNA clones, it is likely that the discrepancy between the apparent mRNA size and the composite sequence in Figure 2 lies in a longer 3' UTR that remains to be isolated.

The mouse *NF2* homologue maps to Chr 11

To determine the chromosomal location of the mouse *NF2* homologue, we sought a polymorphism that would distinguish alleles in C57BL/6 mice and in *M. spretus*, permitting typing of an interspecific backcross. To conserve backcross DNA, we employed single-strand conformational polymorphism (SSCP) analysis using PCR amplification to scan for polymorphism. We first examined two PCR products from the 3' UTR, including the poly(CT) stretch (KM3840 and KM3839; KM4253 and KM3794). Neither of these displayed SSCP, indicating an unexpectedly low level of sequence variation. We then designed primers for the edges of individual exons based on the organization of the human gene (20). Exons 3 (bp 251-373), 8 (bp 686-820), 11 (bp 1010-1132), 12 (bp 1133-1350) and 15 (bp 1585-1750) all amplified successfully, but only exon 12 displayed a useful SSCP pattern (Figure 6). This polymorphism

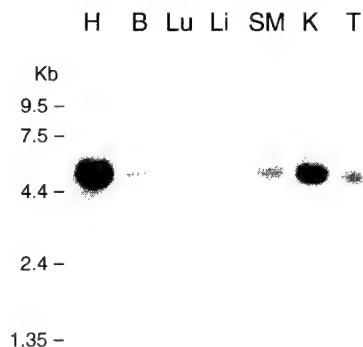


Figure 5. Northern blot survey of mouse *merlin* expression. A Northern blot containing 2 μ g of oligo(dT) selected DNA from various adult mouse tissues was hybridized with a mouse *NF2* cDNA probe. The positions of size markers are shown on the left. H, heart; B, brain; Lu, lung; Li, liver; SM, skeletal muscle; K, kidney; T, testis.

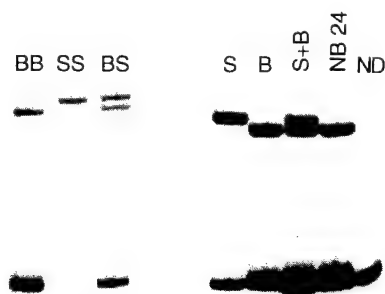


Figure 6. SSCP polymorphism in the mouse *NF2* gene. Exon 12 (based on comparison with the human exon structure) of the mouse *NF2* gene was amplified using primers KM4336 and KM4337 from the exon edges and the products were analyzed for SSCP. The right-hand panel shows the pattern observed for amplification products from *M. spretus* (S), C57BL/6 (B), a mixture of DNA from these two parental strains (S+B) and cDNA clone NB24. The lane labeled ND contains nondenatured PCR product from NB24. The panel on the left shows the patterns detected in the three types of F_2 progeny from the EUCIB, homozygous C57BL/6 (BB), homozygous *M. spretus* (SS) and a C57BL/6/*M. spretus* heterozygote (BS).

was used to type a random sampling of 43 progeny of the European Collaborative Interspecific Backcross panel, which has been typed previously for index markers across the mouse genome. The results of this analysis revealed that the murine *NF2* gene maps to the proximal portion of mouse Chr 11 based on tight linkage with *D11Mit16* (lod score = 10.88 at 2.33 cM).

DISCUSSION

The recent discovery of the gene causing NF2 has uncovered a new type of tumor suppressor. The *merlin* protein is a new member of a class of proteins that have been postulated to act as links between integral membrane proteins and components of the cytoskeleton. The members of the family share a common structure, with a globular N-terminal domain followed by a long

α -helical section and a charged C-terminus. The prototype is erythrocyte protein 4.1 which binds via the N-terminal domain to glycophorin in the membrane and via the α -helical domain to spectrin in the cytoskeleton. *Merlin* is most closely related to three other family members, moesin, ezrin and radixin, which have been found beneath the membrane in areas of cell attachment and membrane protrusion or remodeling. All three of these proteins have previously been cloned in the mouse, and display 46–47% sequence identity with mouse *merlin*, with the similarity concentrated in the N-terminal half of the protein.

The precise function of the *merlin* protein is not yet known, but the near identity of human and mouse *merlin* suggests strong evolutionary constraints on its sequence. Indeed, the sequence conservation extends to the first 100 bp of the 3' UTR, suggesting a potential regulatory function for this segment. On the other hand, the two alternative splices described for mouse *merlin* are predicted to have major effects on the structure of the protein suggesting that *merlin* may play several different roles. The absence of exon 8 from the mRNA removes 45 amino acids from a segment that is identical in both mouse and human proteins, and is 71–74% identical in mouse moesin, ezrin and radixin. In humans, the mRNA transcript missing exon 8 was recently detected as the sole remaining product of the *NF2* gene in a sporadic vestibular schwannoma, suggesting a somatic mutation affecting the splice acceptor site or deleting the exon (21). No genomic DNA was available from the tumor to confirm this supposition, but the absence of any mRNA containing exon 8 from this schwannoma suggests that the 45 amino acids encoded by this exon are critical for the tumor suppressor function of *merlin*. The second alternative splice, which has also been detected in humans, changes the C-terminus of *merlin*, making it more hydrophilic (20,21). Neither of the two different C-termini appears closely related to moesin, ezrin or radixin, for which no alternative splicing has yet been described. The combination of both alternative splices in the *NF2* gene would predict that there are four possible *merlin* isoforms, and it is conceivable that additional alternative splicing will be identified. If this structural variation occurs because *merlin* has several distinct but related functions, then delineating which function must be lost to trigger tumor growth may be complicated.

The high level of evolutionary conservation of the *NF2* gene between mouse and humans is paralleled by a surprisingly low rate of polymorphism. Using SSCP analysis, which is sensitive to most single base pair differences, we were unable to detect differences in the 3' UTR and in most exons tested between the two mouse species. Similarly, scanning of the human exons for mutations in NF2 patients and their tumors has revealed very few polymorphisms. The mouse *NF2* homologue maps to Chr 11, showing only one crossover with *D11Mit16*. Proximal Chr 11 has previously been identified as a region of synteny conservation with the central portion of human 22q, containing the homologues of two genes which flank the *NF2* locus, *NEFH*, the neurofilament heavy chain gene and *LIF*, the leukemia inhibitory factor gene. However, there are no mouse mutants that have been mapped to this region which could be assessed as potential models of NF2. The creation of a mouse model of NF2 using targeted inactivation of the gene in ES cells is the strategy most likely to generate an accurate mouse model of the disorder. The delineation of the mouse *NF2* sequence and patterns of alternative splicing presented here, combined with the exon structure of its highly conserved human homologue should accelerate efforts to create such a model, permitting a detailed

assessment of the mechanism by which *merlin* suppresses tumor formation.

MATERIALS AND METHODS

Isolation and characterization of cDNAs

A randomly primed C1300 mouse neuroblastoma cDNA library (22) was screened under low stringency conditions with either the 5' 1.1 kb *Hind*III fragment or 620 bp internal *Hind*III fragment of the human JJR-1 cDNA clone. Preparation of first strand cDNA, PCR amplification and direct sequencing were performed as described (23). The locations of all specific primers are underlined in Figure 2. The 5'-most mouse coding sequence was obtained using the RACE method (GIBCO BRL). First strand cDNA was made from C1300 total mRNA with primer KM3534 (5'CAGCCTGTTCTCAGGGTC3'). A 3' poly(dC) tail was added to first strand cDNA with terminal deoxynucleotidyl transferase (TdT) (GIBCO BRL). The ~500 bp fragment was amplified by PCR with primer KM3757 (5'GCTTGTGCACAGAGGGGTCATAGTC3') and a poly(G) anchor primer (GIBCO BRL). The PCR product was sequentially reamplified with the nested primers KM3745 (5'AGTGTTCGCTGATCTCTTGA3') and KM4163 (5'GATTGTATACTGCAGTCC3') and the universal amplification primer (GIBCO BRL). The PCR product was subcloned in pBSKII. Several subclones were sequenced. The sequence of all isolated cDNA clones was determined with universal and custom-made oligonucleotide primers by the dideoxy chain termination method as described (24). All PCR amplifications were performed using Taq polymerase (Boehringer Mannheim) under the standard conditions suggested by the manufacturer, with annealing temperatures from 55°C to 60°C and a total of 30 cycles of the following program: 1 min @ 94°C; 2 min @ annealing temp; 3 min @ 72°C.

Northern blots containing poly(A)⁺ RNA from adult mouse tissues were purchased from Clontech Inc. and hybridized under the conditions suggested by the manufacturer with a probe spanning bp 708–1611.

Chromosomal localization

Exon 12 (218 bp) was amplified as described above, using KM4336 (5'CTCCTCTCTGACTCCTCAGCCA3') and KM4337 (5'ATGCGCTCTGAGGAGACAGCTG3') at an annealing temperature of 55°C. SSCP was performed at room temperature as described using an 8% nondenaturing polyacrylamide gel containing 8% glycerol. (25,26). The SSCP variant shown in Figure 6 was typed in 43 animals from the European Collaborative Interspecific Backcross, a reference mapping resource that consists of 1,000 F₂ progeny, 500 generated by backcrossing the F₁ *M.spretus*/C57BL/6 heterozygotes to each of the parental strains. This shared resource has been typed for index markers across the mouse map to facilitate placing any new locus, such as *NF2*, within a genetic interval.

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Exon scanning for mutation of the *NF2* gene in schwannomas

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Family studies and tumor analyses have combined to indicate that neurofibromatosis 2 (NF2), a disorder characterized by multiple benign tumors of the nervous system, and sporadic non-inherited forms of the same tumor types are both caused by inactivation of a tumor suppressor gene located in 22q12. Recently, the gene encoding *merlin*, a novel member of a family of cytoskeleton-associated proteins, was identified as the NF2 tumor suppressor. To facilitate the search for *merlin* mutations, we have defined the exon–intron boundaries for all 17 NF2 exons, including one subject to alternative splicing. We have developed polymerase chain reaction assays to amplify each exon from genomic DNA, and used these assays to perform single-strand conformation polymorphism analysis of DNA from 30 sporadic and eight NF2-derived schwannomas, the hallmark tumor type in this disorder. Of a maximum of 60 alleles scanned, 32 showed mutations affecting expression of the *merlin* protein. Thirty of these mutations are predicted to lead to a truncated protein due to frameshift, creation of a stop codon, or interference with normal splicing, while two are missense mutations. Thus, inactivation of *merlin* is a common feature underlying both inherited and sporadic forms of schwannoma.

INTRODUCTION

Neurofibromatosis 2 (NF2) is a severe inherited disorder affecting approximately 1 in 40,000 individuals and characterized by the formation of multiple benign nervous system tumors (1–3). A defining feature of NF2 is the bilateral occurrence of schwannomas on the eighth cranial nerve (vestibular schwannomas), but NF2 patients also suffer from intracranial and peripheral schwannomas, ependymomas and meningiomas. In a subset of cases, posterior subcapsular lenticular opacities are also detected (4). The disorder has a profound impact on its victims, as multiple slow-growing tumors gradually compress adjacent nerves, causing deafness, disrupted balance control, paralysis and early death.

The tumors of NF2, and similar tumors which occur sporadically in the population, have both been proposed to be

associated with inactivation of a tumor suppressor gene located in chromosome band 22q12 (5–17). In NF2 families, a damaged allele is transmitted, but tumor formation presumably only occurs when somatic mutation of the remaining wild-type allele frees the target cells from the actions of the tumor suppressor. Sporadic tumors presumably result from somatic inactivation of both alleles in a single target cell. Using a location cloning strategy, we have recently identified the NF2 gene based on germline mutations in several NF2 patients, including non-overlapping interstitial deletions (18). The disease locus encodes *merlin*, a novel member of a family of proteins that are thought to link membrane proteins with the cytoskeleton. Independent confirmation of this disease gene has subsequently been reported, along with a placement of 16 of its exons (19). To facilitate the search for mutations in NF2 and related tumors, we have sequenced the exon–intron junctions of the NF2 locus, including an additional exon shown to be alternatively spliced into the transcript (20,21). Polymerase chain reaction (PCR) assays were developed for all 17 exons, and were used to scan the entire gene for mutations in both sporadic and inherited vestibular schwannomas. The high proportion of tumors in which inactivating mutations were found indicates that the NF2 gene plays a fundamental role in schwannoma tumorigenesis.

RESULTS

Exon structure of the NF2 gene

The NF2 gene has been reported to consist of 16 exons, encoding the 595 amino acid *merlin* protein (18,19). We have recently demonstrated an alternatively spliced exon (E16) that alters the C-terminus of *merlin*, removing 16 residues and replacing them with 11 novel amino acids (20,21). To provide the basis for scanning the entire gene for mutations in NF2 and related tumors, we have sequenced the intron–exon junctions of all 17 NF2 exons. The internal exons range in size from 45 to 218 bp, with an average of 111 bp. Of the 15 internal exons, 10 (E3, E5–7, E9–11, E14–16) were isolated successfully using exon amplification, the technique which led to isolation of the NF2 locus from cloned genomic DNA (18, Trofatter *et al.*, unpublished). Table 1 shows the DNA sequences immediately surrounding the intron–exon junctions, which in all cases match the consensus for splice acceptor and splice donor sites. Additional intron sequences on both 5' and 3' sides that were used to design primers for PCR amplification can be found in GenBank submissions L27131 through L27147.

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Table 1. Intron-exon boundaries of the *NF2* gene

Exon	Splice acceptor	Start (bp) ¹	Exon start	Exon length	Exon end	End (bp)	Splice donor
1					GAG	114	GTAACCGGCC
2	GTTATTGCAG	115	ATG	126	AAG	240	GTGGGCTAG
3	AATTCTGCAG	241	GTA	123	CAG	363	GTACATCAGT
4	CTCCTTTCAG	364	GTA	84	AAG	447	GTAGGCTCAA
5	TTCTTTCAG	448	TAT	69	AGG	516	GTAAGAGATT
6	TTTITGGTAG	517	GTA	83	CAG	599	GTGAGGCCCA
7	CTCCCCACAG	600	GGA	76	CGG	675	GTGTGTTGAA
8	GGATCCACAG	676	AAT	135	GAG	810	GTAGGACATG
9	ATTCTTCCAG	811	TTT	75	CTG	885	GTAAGTTGAG
10	GTGGCCACAG	886	ATT	114	CAG	999	GTAGGCACAA
11	CCCCTCGCAG	1000	ATG	123	CTG	1122	GTGATTTCTG
12	TGCCCTCCAG	1123	ATG	218	GAG	1340	GTGAGGGGGC
13	TTCCTTGCAG	1341	GCG	106	CCG	1446	GTGAGCTGGG
14	TCATTAACAG	1447	CCC	128	AAA	1574	GTATGTAGCC
15	TTGCCGGCAG	1575	AGT	163	AAG	1737	GTACCCAGGG
16	GCTGGTTTAG	1738	CCT	45	AAA	1782	GTAGGTTGTT
17	TTTCTTACAG	1783	CTC				

¹Base pair numbering is based on #1 being the A of the initiator ATG

Table 2. Primers for exon PCR assays

Exon ¹	Product (bp)	Temp. ² (°C)	Primer #1 (5'–3')	Primer #2 (5'–3')
1	236	58	GCTAAAGGGCTCAGAGTGCAG	GAGAACCTCTCGAGCTTCCAC
2	180	60	TGTCCTTCCCCATTGGTTTG	CAGTTTCATCGAGTTCTAGCC
	243	58	AGTGCAGAGAAAAGGTTTATTAATGAT	TGGAAAGCTCAGTCAGCC
3	275	60	GCTTCTTGAGGGTAGCAC	GGTCAACTCTGAGGCCAACT
4	188	59	CCTCACTTCCCCTCACAGAG	CCCATGACCCAAATTAACGC
5	170	60	GCTCTCCCTTCTCTTTCC	TCCTTCAAGTCTTTGGTTAGC
	172	58	TGGCAGTTATCTTTAGAATCTC	TTAGACCACATATCTGCTATG
6	161	60	CATGTGTAGGTTTTTATTTTGC	GCCCATAAAGGAATGTAACC
7	174	60	CCATCTCACTTAGCTCCAATG	CTCACTCAGTCTCTGTCTAC
	171	60	GAATGCTTGATTTGGTGCC ³	GAGGTTTCAACACACCCGGA
8	248	60	GAAGGTTGAATAAAATTTTGGCCTC	GACAGGGAAGATCTGCTGGACC
	293	60	CTGTTCTTATTGGATCCACAG	AACAACCAACCCCTCAAGC
9	300	58	GACTTGGTGCTCCTAATTCCTC	CCATTATCAGTAATGAAAACCAGG
10	260	59	TGCTACCTGCAAGAGCTCAA	CTGACCAACAGTGACATC
11	268	60	TCTTTGGCCCTTGTGGCAC	CAGGAGACCAAGCTCCAGAA
12A	137	60	TTCAAGTAAGAGCACTGTGC	CGTGCATTTCTGCTCAG
12B	284	58	GCTGAAAAGGCCAGATCA	CTTGAGGACAAGTCTGTAG
13	227	60	GGTGTCTTTCTGCTACCT	GGGAGGAAAGAGAACATCAC
14	253	60	TGTGCCATTGCCTCTGTG	AGGGCACAGGGGGCTACA
15	317	58	TGGCCAAGTAGAGACGTGA	TACAAGAAAGAGACCTGGG
	244	58	TCTGCCCAAGCCCTGATGC	TGGTCTGATCAGCAAAATAC
16	148	60	GGCATTGTTGATATCACAGG	GGCAGCACCATCACACATA
17	178	60	CTCTCAGCTTCTTCTGTCT	CCAGCCAGCTCTATGGATG ³

¹All exons were scanned by single PCR assays except exon 12, where overlapping assays (12A and 12B) were required. For exons 2, 5, 7, 8, and 15, two different PCR assays were developed.

²Annealing temperature for PCR reaction.

³These primers, synthesized before the final sequence was perfected, are missing a single base relative to the genomic sequence.

PCR assays for the 17 *NF2* gene exons

For each internal exon, primers were chosen in flanking intron sequences to develop an assay for PCR amplification of the exon directly from genomic DNA. For E12, two overlapping primer sets were chosen to yield products in a size range amenable to single-strand conformation polymorphism (SSCP) analysis. All of the coding region from exon 1 is amplified using one primer in the 5' untranslated region (UTR) and one in intron 1. For exon 17, a primer from the final intron is paired with a primer in the 3' UTR to amplify all of the exon 17 coding sequence, along with the first 100 bp of 3' UTR, which shows sequence conservation in the mouse (21). All assays except that for exon

1 are run under a standardized set of amplification conditions, varying only the annealing temperature to optimize results. Table 2 lists the primers, annealing temperature and product size for each exon assay. In some cases (E7 and E8), an initial set of primers was used, but subsequently was replaced with a second primer pair. In these instances both sets of primer pairs are listed.

Scanning for mutations by SSCP analysis of blood-tumor pairs

We have used the exon PCR assays listed in Table 2 to scan the entire *merlin* coding sequence for mutations in schwannomas. SSCP analysis was applied to DNA extracted from 38 primary

Table 3. *NF2* gene mutations in schwannomas from NF2 patients

Tumor	Exon	DNA sequence alteration ¹	Codon change ²	Consequence	Origin ³	Alleles ⁴
S1	E7 E10	655 G→A 904/6 to 931/3 del 28 bp	Val219Met Gly302fs > 322X	Missense Frameshift	S G	2
S4	E3	353 to 363+19 del 30 bp		Splice donor site	S	2
S10	E13 E9	1396 C→T 844 del 1 bp (G)	Arg466X Val282fs > 296X	Nonsense Frameshift	G S	2
S11	E2	169 C→T	Arg57X	Nonsense	G	2
S32	E12	1340+2 ins 1 bp (t)		Splice donor?	G	2
S33	E2	179 G→A	Trp60X	Nonsense	G	1
S34	E14	1451 to 1452 del 2 bp (TG)	Met484fs > 494X	Frameshift	S	NI
S36	E7	600-28 to -5 del 24 bp		Splice acceptor?	S	2

¹Numbering of bases showing alteration is given relative to the cDNA sequence with the initiator ATG beginning at base 1. All coding sequence bases are given in upper case. When the alteration affects intronic sequence, it is presented in lower case and numbered as '-' (5' intron) or '+' (3' intron) the requisite number of bases from the first or last base of the exon, respectively. For deletions, the span of deleted bases (numbered as above) is given, followed by the deletion size ('del'). For deletions of less than 5 bp, the deleted base are also named. Where the start position of the deletion is uncertain, the alternative ranges of bases deleted are shown. Insertion is indicated by 'ins' followed by the number of bases inserted, and their identity.

²Original amino acid and position of the residues in the protein (numbered from the initiator Met as 1) is followed by new amino acid for missense mutation, X for nonsense mutation, or fs for frameshift, followed by the position of the next in-frame stop codon.

³S = somatic mutation; G = germline mutation.

⁴Number of *NF2* alleles in tumor predicted by heterozygosity testing with Chr 22 DNA markers. NI = not informative.

Table 4. *NF2* gene mutations in sporadic schwannomas¹

Tumor	Exon	DNA sequence alteration	Codon change	Consequence	Origin	Alleles
S2	E3	241-22 to -13 del 10 bp		Splice acceptor?	S	1
S3						NI
S5						1
S6						2
S9	E7	600-32 t→a		Acceptor-branch site?	G	2
S12	E7	600-1 g→a		Splice acceptor site	S	1
S13						2
S14						1
S15	E15	1634/6 to 1635/7 del 1 bp (A)	Ile546fs > 550X	Frameshift	S	1
S16	E4	439 del 1 bp (C)	Gln147fs > 174X	Frameshift	S	2
S17						2
S18	E8	676-10 to 726 del 61 bp		Splice acceptor site	S	2
	E12	1266 to 1267 del 2 bp (GA)	Glu422fs > 442X	Frameshift	S	
S19	E15	1575-26/-27 to 1581/2 del 34 bp		Splice acceptor site	S	1
S22	E7	675+1 g→t		Splice donor site	S	1
S23						1
S24	E7	634 C→T	Gln212X	Nonsense	S	2
	E10	905 to 912 del 8 bp	Gly302fs > 331X	Frameshift	S	
S25	E10	992 to 999+1 del 9 bp		Splice donor site	S	1
S26						1
S27						2
S29	E2	134 del 1 bp (A)	Asp45fs > 123X	Frameshift	S	2
	E8	729 to 732 del 4 bp (TTAT)	Ile243fs > 251X	Frameshift	S	
S30	E4	447 or 447+1 del 1 bp (G or g)	Lys149fs > 174X	Frameshift or splice donor site	S	1
S31						1
S35	E1	65/70 to 79/84 del 15 bp 88 to 109 del 22 bp	del 5 aa Asp30fs > 40X	Frameshift	S	1
S37	E4	364-2 a→g		Splice acceptor site	S	2
S38	E10	933 del 1 bp (G)	Arg311fs > 322X	Frameshift	S	2
S39	E12	1223 to 1227 del 5 bp	Glu408fs > 442X	Frameshift	S	1
S40						1
S42	E14	1517/20 ins 1 bp (T)	Phe507fs > 513X	Frameshift	S	2
S43	E14	1571/4 del 1 bp (A)	Lys525fs > 550X	Frameshift	S	2
S44	E12	1252 C→T	Arg418Cys	Missense	G	2

¹Explanation of all symbols can be found in Table 3.

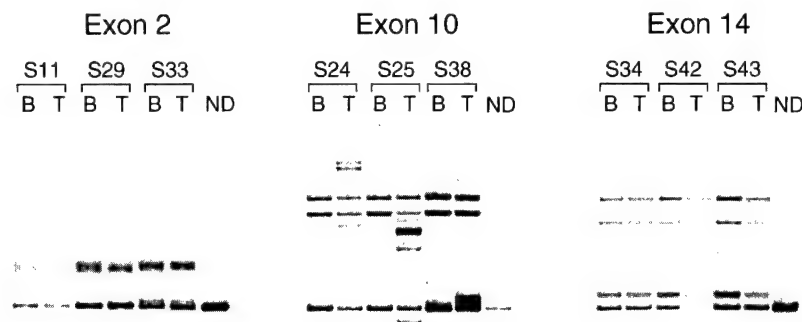


Figure 1. Representative SSCP analyses. For each exon, control blood (B) and tumor (T) DNAs were analyzed and a non-denatured sample (ND) was included. Abnormal migration patterns are observed for each tumor DNA. Two blood DNAs from NF2 patients, S11 and S33, show the same alterations in exon 2 as the tumor DNAs, suggesting that these shifts represent germline mutations.

tumor specimens, including eight vestibular schwannomas from NF2 patients (Table 3), 27 sporadic vestibular schwannomas (Table 4) and three sporadic spinal schwannomas (Table 4: S9, S25 and S27). DNA extracted from a blood sample of the corresponding patient was used for comparison in each case. All tumor-blood pairs were assayed for all exons.

Representative results of the SSCP analyses for three of the exons, E2, E10 and E14 are shown in Figure 1. For each assay, a lane of PCR product was run without denaturation (ND) to identify fully reannealed, double-stranded DNA in the test lanes. The other bands in the test lanes represent various conformations of the single-stranded DNAs in the product. For E2, the normal pattern is seen only in the blood DNA (B) of S29. S11 and S33 display mobility shifts that are detected in both blood and tumor DNA, indicative of germline alterations. S29 displays a mobility shift only in the tumor DNA, indicating a somatic mutation. For E10 and E14, all blood DNAs display the normal pattern and all tumor DNAs display different mobility shifts. Both normal and altered PCR products were compared by direct sequence analysis to identify the precise base change(s) involved.

NF2 mutations in schwannomas

The blood and tumor DNAs were genotyped using polymorphic DNA markers (Tables 3 and 4) to detect loss of heterozygosity that could indicate deletion of one *NF2* allele. Overall, the heterozygosity testing predicted that at least 16 of the 38 tumors had lost one *NF2* allele (two tumors were not informative for heterozygosity testing). Thus, our SSCP scanning examined the entire *NF2* coding sequence for 58–60 independent alleles, eight of which are predicted to possess germline mutations. Despite the large number of alleles examined, we identified no obvious polymorphisms affecting either coding or non-coding sequences, indicating a remarkable degree of homogeneity for this gene sequence in the population. By contrast, mutations in both NF2 and sporadic tumors were readily detected. To date, DNA differences from the normal sequence have been confirmed in 27 of these tumors, with seven germline and 25 somatic alterations identified by sequence analysis. These results are summarized in Tables 3 and 4.

Germline mutations, present in both blood and tumor DNA, were delineated in five of the eight patients with a confirmed diagnosis of NF2 (Table 3). These changes occurred in disparate locations, including point mutations leading to stop codons at

residues 57 and 60 of E2 (S11 and S33) and 1396 of E13 (S4), a 28 bp deletion leading to a frameshift and premature stop codon in E10 (S1), and an insertion of one base into a splice donor site in E12 (S32). Two other germline alterations were found in blood and tumor DNA from individuals without a confirmed NF2 diagnosis (Table 4), a single base change in the intron upstream of E7 in a sporadic spinal schwannoma (S9) and a substitution of Cys for Arg at residue 418 in a sporadic vestibular schwannoma (S44). Although the two intron changes and the apparent missense mutation could conceivably represent polymorphisms, they were not found by SSCP analysis of 150 independent DNA samples from normal individuals or individuals with other types of tumors.

Somatic mutations were observed in tumors from five of the eight patients with NF2, including two (S1 and S4) for whom a germline mutation was also detected (Table 3). In the sporadic tumors, a total of 20 somatic mutations were found in 30 tumors (Table 4). In three cases where heterozygosity for chromosome 22 was maintained (S18, S24, and S29), two distinct somatic alterations were found in each tumor. For example, tumor S29 displayed small deletions of 1 bp and 4 bp in E2 and E8, respectively (Figure 2). In one tumor (S35), a complex of two adjacent deletions was detected in a single allele, with one loss removing 5 codons in-frame, and the other, beginning 3 bp downstream, causing a frameshift.

The 25 somatic mutations from both NF2 and sporadic tumors were found throughout the gene, associated with exons E1, E2, E3, E4, E7, E8, E9, E10, E12, E14 and E15. By far the most frequent lesions detected (19 of 25) were small deletions of 1–61 bp, that have either an obvious or presumed effect on splicing or that produce frameshifts leading to truncated proteins of altered sequence. A single mutation involved a frameshift due to a single base insertion (S42). The remaining five somatic changes were all point mutations that either altered splice donor (S22) or acceptor sites (S12, S37), produced a stop codon at residue 212 (S24) or generated a Met for Val substitution at residue 219 (S1).

DISCUSSION

NF2 is a disorder that matches closely the expectations for a 'two-hit' model of tumorigenesis, in which homozygous inactivation of a gene that normally suppresses tumor growth is the critical

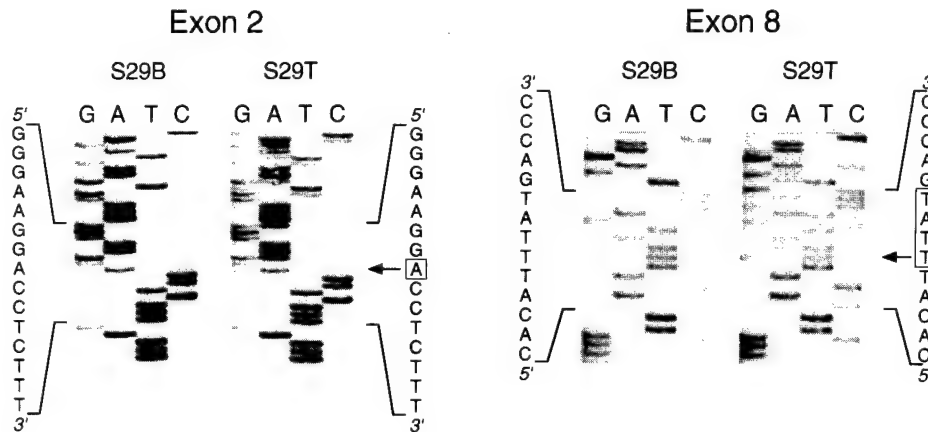


Figure 2. Direct DNA sequence analysis of two alterations in S29. The DNA sequences surrounding the sites of two mutations are shown for tumor DNA (T) and control blood DNA (B) from patient S29. The tumor is heterozygous for each of a 1 bp deletion in exon 2 and a 4 bp deletion in exon 8 (shown in boxes). These deletions are predicted to generate premature stop codons at positions 123 and 251, respectively.

initiating event in tumor formation (22). The same types of tumors that are present as multiple independent growths in NF2 patients occur as sporadic, solitary cases in the general population (1,2). The familial and sporadic tumors both display frequent loss of genetic material from chromosome 22, in a region to which the *NF2* gene defect has been mapped by linkage analysis (4–17). Thus, it is presumed that the *NF2* locus encodes a tumor suppressor and that inactivation of both alleles by loss or mutation in specific cells results in uncontrolled proliferation. However, only specific cell types are affected in this way by inactivation of this tumor suppressor, since the vast majority of tumors seen in NF2 are schwannomas (particularly vestibular schwannomas) and meningiomas.

The recent cloning of the *NF2* gene has provided evidence for germline mutations in the coding sequence for *merlin*, a novel member of a family of cytoskeleton-associated proteins, implicating this protein as a tumor suppressor (18,19). Preliminary analysis of NF2 patients for mutations affecting *merlin* has revealed both large and small deletions, point mutations affecting splice sites or creating stop codons, and at least two missense mutations, Leu360Pro and Asn220Tyr (18,19,23). To date, there have been only limited data on somatic mutation at the *NF2* locus. Examination of the *merlin* mRNA by reverse transcription PCR has provided evidence for splicing mutations or deletions in six sporadic and two germline vestibular schwannomas (20). Analysis of genomic DNA has yielded nonsense mutations in two sporadic schwannomas, and frameshift mutations in two sporadic meningiomas (19).

The data presented here confirm a critical tenet of the tumor suppressor model in NF2. Although germline mutations are present in both blood and tumor DNA from NF2 patients, somatic mutation affecting expression of *merlin* is clearly a frequent event in both inherited and sporadic schwannomas. In several cases, two inactivating mutations were detected in the same tumor while in many others a single mutant allele remained due to loss of the second copy of the locus. The alterations occurred throughout the *NF2* gene, with most exons displaying at least one mutation, although we have yet to identify a change within either the normal C-terminus (E17) or that produced by alternative splicing (E16).

One alteration, conversion of the Arg codon at position 57 to a stop codon, has been seen twice before in independent NF2

patients (19), suggesting that this site containing a CpG dinucleotide may be particularly prone to C to T transitions. The presence of this change in the blood DNA of S11 combined with its absence from either parent demonstrate that this is a case of new mutation to NF2. Although many different frameshift and nonsense mutations were seen, a surprising number of somatic mutations involved changes in the intron sequences, sometimes at a distance from the exon–intron junction. While we presume that these affect proper splicing, no RNA is available from these tumors to confirm this supposition. However, the absence of these alterations from blood-derived DNA of the same individuals, and the failure to detect the same change in any other individuals indicate clearly that these mutations are *de novo* events associated with tumor formation.

Two new missense mutations were identified, Val219Met and Arg418Cys, which may target these residues as particularly important in *merlin*'s tumor suppressor function. The Val at position 219, one residue away from the previously reported Asn220Tyr mutation, is located within the protein 4.1 domain that is characteristic of this family and is conserved in human moesin, radixin and protein 4.1, but changed to Ile in human ezrin. The Arg at position 418 is located in the long α -helical domain that comprises most of the C-terminal half of *merlin* and its relatives, but is not strictly conserved in the other human members of this protein family. However, both Val219 and Arg418 are conserved in mouse *merlin* (21).

In addition to expanding the number and variety of germline NF2 mutations described, these studies have yielded intriguing suggestions of germline alterations in patients not recognized clinically as suffering from NF2. One patient, a female aged 37 years with a single spinal schwannoma, displays a single base alteration in intron sequences, while the other, a 61 year old male with a single vestibular schwannoma, possesses the Arg418Cys change described above. Careful clinical follow-up is indicated for these patients to determine whether additional stigmata of NF2 become evident with time. It is possible that they represent a class of individuals with mutations that only mildly affect normal *merlin* function, and consequently do not produce the full NF2 phenotype.

The strategy employed in this study, PCR amplification of each exon in the *NF2* gene combined with SSCP analysis identified

about half of the potential mutations, 32 of a possible 58–60. The failure to identify mutations in 100% of cases is likely due to several causes. First, we carried out SSCP analysis under a single set of conditions. It is very likely that varying SSCP conditions to increase sensitivity, or employing a complementary mutation scanning technique, such as denaturing gradient gel electrophoresis or chemical cleavage of mismatches, would identify additional alterations. Second, it is likely that some of the tumors that remain heterozygous for flanking chromosome 22 markers have in fact suffered smaller interstitial deletions that remove either the whole *NF2* gene, or one or more exons. Such deletions, which have already been demonstrated in several NF2 patients, would not be detected by our PCR approach. Third, some mutations may lie outside of the regions amplified, either in 5' regulatory elements, further into the introns, in the 3' UTR, or in additional alternatively spliced exons not yet identified. Fourth, some tumors might result from a combination of reduced function of the *NF2* gene with a lesion at another locus, rather than complete inactivation of *merlin*. Finally, it is conceivable that some of the somatic mutations might be dominant negatives whereby the altered *merlin* product acts to interfere with and thereby inactivate its remaining normal counterpart.

The development of reliable PCR assays for each exon of the *NF2* gene should facilitate greatly the cataloging of mutations in NF2 patients and their tumors by genomic scanning. It can be expected that a detailed mutational analysis of the *NF2* gene, identifying sites particularly prone to alteration, pinpointing amino acid residues crucial for normal function, and providing a basis for relating specific alterations with variations in phenotype will result. Perhaps most important, however, the ability to rapidly scan the *NF2* gene for mutation will accelerate the assessment of a role for *merlin* in other tumor types, indicated by recent findings of mutations in breast cancer and melanoma (20).

MATERIALS AND METHODS

Tissue samples

After obtaining informed consent, tumor specimens were obtained at the time of surgery and frozen for DNA analysis. Blood samples were also obtained at the time of surgery to serve as normal tissue controls. High molecular weight DNA was extracted from peripheral blood leukocytes and from frozen pulverized tumor tissue by SDS-proteinase K digestion followed by phenol and chloroform extractions (9).

Design of primer pairs

Exonic primers were designed within the *NF2* coding sequence near the intron–exon borders as determined by the results of exon trapping (18). For those regions not isolated by trapping, primers were synthesized at approximate 100 bp intervals. Using these primers, intronic sequence was obtained by directly sequencing cosmid containing the gene using a cycle sequencing kit (US Biochemical). Intronic primer pairs were then designed to amplify the splice donor and acceptor sites as well as the exon itself. In a single case (E12) it was necessary to construct two overlapping primer sets to maintain a product length of less than 300 bp.

SSCP analysis

SSCP analysis was performed according to the procedure of Orita *et al.* (24) with minor modifications. Approximately 50 ng of genomic DNA was amplified using appropriate intronic primer pairs (Table 2). Each 10 μ l reaction contained 70 μ M each of dATP, dCTP, dGTP and dTTP, 4 pmol of each primer, 0.5 units Taq polymerase, 10 mM Tris pH 8.3, 1.5 mM MgCl₂, 50 mM KCl, 0.01% gelatin, and 0.1 μ l α -[³²P]dATP (Amersham, 10 mCi/ml). For exon 1, a MgCl₂ concentration of 0.5 mM was used. Amplification was carried out for 30 cycles as follows: 94°C for 1 min, 55–60°C for 1 min, and 72°C for 1 min, after an initial denaturation step at 94°C for 4 min. One μ l of labeled amplified DNA was diluted into 9 μ l 0.1% SDS and 10 mM EDTA, and an equal volume of loading dye (95% formamide, 0.5 M EDTA, 0.05% bromophenol blue and 0.05%

xylene cyanol) was added. The samples were denatured for 2 min at 90°C and separated on 6–8% polyacrylamide gels containing 8% glycerol for 16 h at 6–8 W. Gels were dried and exposed to Kodak X-OMAT film.

DNA sequencing

For DNA sequencing PCR amplifications were performed in 50 μ l volumes as described above for SSCP analysis except for the addition of 200 μ M dNTPs and the omission of a radioactive nucleotide. This product was sequenced by one of two methods. In the first, the double-stranded product was used as a template to generate single strands by priming multiple rounds of DNA synthesis with one of the oligonucleotides previously used in the double-strand reaction (25). Conditions for asymmetric PCR amplification were identical except that only one primer was added. The product was ethanol precipitated in the presence of ammonium acetate and resuspended in 10 μ l water for subsequent sequence analysis. The sequencing reactions were performed by the dideoxy chain-termination method using Sequenase (T7 DNA polymerase, US Biochemical) under conditions recommended by the supplier. Alternatively, the PCR products were purified with BioSpin columns (BioRad), ethanol precipitated, DNA sequencing was performed according to a standard cycle-sequencing protocol using VentR (exo-) DNA polymerase and the CircumVent Thermal Cycle kit (New England BioLabs). Both strands of exons with SSCP mobility shifts were analyzed in all cases.

Loss of heterozygosity analysis

Genomic DNA was amplified using primer pairs for the polymorphic dinucleotide repeats at markers *D22S193* (Trofatter *et al.*, in preparation) or *D22S268* (26). The CA-strand primer was 5' end-labeled with polynucleotide kinase and γ -³²P ATP and PCR was performed as described previously (27). In some cases, loss of chromosome 22 alleles was determined by Southern blot analysis using probes at the following loci: *D22S22*, *D22S29*, *D22S28*, *D22S15*, *D22S1*, *CRYB2*, *D22S10* and *D22S9* (28).

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Mutational Analysis of Patients with Neurofibromatosis 2

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Summary

Neurofibromatosis 2 (NF2) is a genetic disorder characterized by the development of multiple nervous-system tumors in young adulthood. The NF2 gene has recently been isolated and found to encode a new member of the protein 4.1 family of cytoskeletal associated proteins, which we have named *merlin*. To define the molecular basis of NF2 in affected individuals, we have used SSCP analysis to scan the exons of the NF2 gene from 33 unrelated patients with NF2. Twenty unique SSCP variants were seen in 21 patients; 10 of these individuals were known to be the only affected person in their kindred, while 7 had at least one other known affected relative. In all cases in which family members were available, the SSCP variant segregated with the disease; comparison of sporadic cases with their parents confirmed the de novo variants. DNA sequence analysis revealed that 19 of the 20 variants observed are predicted to lead to a truncated protein due to frameshift, creation of a stop codon, or interference with normal RNA splicing. A single patient carried a 3-bp deletion removing a phenylalanine residue. We conclude that the majority of NF2 patients carry an inactivating mutation of the NF2 gene and that neutral polymorphism in the gene is rare.

Introduction

Neurofibromatosis 2 (NF2) or central neurofibromatosis is a genetic disorder characterized by bilateral vestibular schwannomas (formerly called *acoustic neuromas*), schwannomas of other cranial and peripheral nerves, meningiomas, and ependymomas (Martuza and Eldridge 1988). It is inherited in an autosomal dominant fashion with full penetrance; one half of all cases have no previous family history and are felt to be due to new mutations (Ev-

ans et al. 1992). Its overall incidence is ~1 in 40,000 individuals, and there is no known ethnic predilection. Affected individuals generally develop symptoms of eighth-nerve dysfunction in early adulthood, including deafness and balance disorder. Occasionally onset is in childhood or delayed until the 5th or 6th decade. Although the tumors of NF2 are histologically benign, their anatomic location makes management difficult, and patients suffer great morbidity and mortality.

Because the tumors of NF2 patients and similar tumors that occur sporadically in the population have both been associated with loss of chromosome 22 material, the NF2 gene was postulated to be a classic tumor suppressor. Recently we and others have identified the NF2 gene and have shown its inactivation in both germ-line and tumor material, supporting this hypothesis (Rouleau et al. 1993; Rubio et al. 1993; Trofatter et al. 1993; Jacoby et al. 1994). The protein product, which we have named *merlin* (for *moesin-ezrin-radixin-like protein*), shows an unexpected but close relationship to a family of well-studied cytoskeleton-associated proteins. The same protein was subsequently named *schwannomin* by Rouleau et al. (1993). Members of this family are conserved throughout mammalian species and function in a number of roles including maintenance of membrane stability by the binding of integral membrane proteins and the spectrin actin cytoskeleton. The identification of a member of this family as a tumor suppressor suggests a role for integrity of cytoskeleton-membrane protein interactions in growth control.

To delineate more precisely the spectrum of NF2 mutations in germ-line and somatic cells, we have defined the intron/exon junctions within the NF2 locus and have designed PCR assays for all 17 known exons (Jacoby et al. 1994). In the present report we describe the use of these assays to scan DNA samples from 33 affected unrelated individuals and their families, for both neutral and disease-related variants, using SSCP analysis in genomic DNA. When possible, we have tracked these alterations through pedigrees to show segregation with the affected state. We have also determined the DNA sequence and the predicted effects on the protein product of the NF2 gene in these patients. The results confirm that mutations producing

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structural alterations of the merlin protein are frequent in both familial and sporadic NF2 cases.

Patients, Material, and Methods

NF2 Patients

As part of ongoing efforts to define the clinical and molecular characteristics of NF2, a large number of affected individuals and their relatives have been recruited through private physicians and patient-support groups by the Massachusetts General Hospital and the National Cancer Institute. Our scan for mutations began with a cohort of 33 patients. Fourteen of these patients were not known to have any affected relatives, 12 had at least one other affected relative, and 7 had been lost to follow-up by the time of this study. Four patients are part of previously described kindreds. Individuals 5434, 6272, and 7861 are affected members of pedigrees 6, 9, and 12, respectively, described by Narod et al. (1992). Individual 13940 is an affected member of the large kindred described by Short et al. (1994). As this work progressed, germ-line mutations were found in tumors from individuals 13158 and 4974 (specimens S11 and S32, respectively, in Jacoby et al. 1994). Criteria used to determine affected and unaffected status were those of the NIH (Mulvihill et al. 1990), as modified by Evans et al. (1992). This study was approved by the appropriate institutional review boards, and informed consent was obtained from all patients and family members.

Lymphoblast Lines and DNA Extraction

For the majority of patients and family members, lymphoblast lines were established from peripheral blood samples as described elsewhere (Anderson and Gusella 1984). DNA was isolated from peripheral or cultured leukocytes as described elsewhere (Jacoby et al. 1994).

Oligonucleotide Primers and SSCP Analysis

Oligonucleotide primers were designed to amplify the 17 known exons of the NF2 gene, as well as the adjacent splice junctions (Jacoby et al. 1994). In addition to the entire coding sequence, this primer set amplifies 60 bp of the 5' UTR and 98 bp of the 3' UTR. SSCP analysis was performed according to the procedure of Orita et al. (1989), with the following modifications. Approximately 30 ng of genomic DNA was amplified using appropriate intronic primer pairs. Each 10- μ l reaction contained 20 μ M each of dATP, dGTP, dCTP, and dTTP, 4 pmol of each primer, 0.5 units of *Taq* polymerase, 10 mM KCl, 0.1 mg gelatin/ml, and 0.1 μ l of either alpha [33 P]- or [32 P]-dATP (10 mCi/ml; Amersham). For exon 1, a $MgCl_2$ concentration of 0.5 mM was used. Each reaction was cycled as described elsewhere (Jacoby et al. 1994). The amplified DNA was diluted with 20 μ l of 1.5 M NaOH and 10 μ l of loading dye (95% formamide, 0.5 M EDTA, 0.05% bromophenol blue, and

0.05% xylene cyanol). To confirm positive results in selected samples, 1 μ l of labeled amplified DNA was diluted into 10 μ l of 0.1% SDS and 10 mM EDTA and an equal volume of loading dye. The samples were briefly denatured at 95°C and were kept on ice until separated on 6%–8% polyacrylamide gels containing 8% glycerol at 4°C or room temperature. All samples were run in duplicate under different conditions. Each gel contained several controls including a lane of PCR product run without denaturation to identify fully reannealed, double-stranded DNA, an unaffected individual, and an amplified product from a cosmid clone containing the test exon to identify amplification products from other related loci. When available, a known positive control was also used. Electrophoresis was carried out at 5–10 W constant power for 12–36 h, depending on the size of the fragment amplified. Gels were dried and exposed to Kodak X-OMAT film.

DNA Sequencing

Direct sequencing of both strands of PCR-amplified exons was performed as described elsewhere (Jacoby et al. 1994). In selected cases the amplified products were also cloned in T vector (Novagen), and the DNA obtained from several individual clones was sequenced using a Sequenase kit (USB).

Restriction-Digest Analysis

In selected cases exons were amplified as outlined above, with the omission of the radionucleotide. Ten microliters of the amplified product was added to 4–8 units of an appropriate restriction enzyme and incubated at an appropriate temperature for 1 h. Products were separated on a 3% agarose gel (1% Nu-T Sieve GTG agarose and 2% Seakem agarose [FMC Bioproducts]) and were visualized with ethidium bromide staining.

Results

SSCP Analysis of Affected Individuals

In the current study the entire coding region of the NF2 gene was scanned in a total of 33 affected unrelated individuals by using 18 exon-based assays. A total of 20 SSCP shifts were detected in 11 exons; all samples gave normal patterns for exons 1, 4, 7, 9, 16, and 17. Results typical of the SSCP shifts seen in this work are shown, in figure 1A, for a sporadically affected patient and, in figure 2, for a large kindred. Twelve individuals showed no variation in any of the exons examined. Two unrelated individuals (5434 and 16773) gave identically altered patterns in the same exon.

Of the 21 individuals found to have alterations, 10 were the only affected member of their family, while 7 were known to have other affected family members. Three had been lost to follow-up, and one did not know the status of her biological family. Persons in whom no alteration was

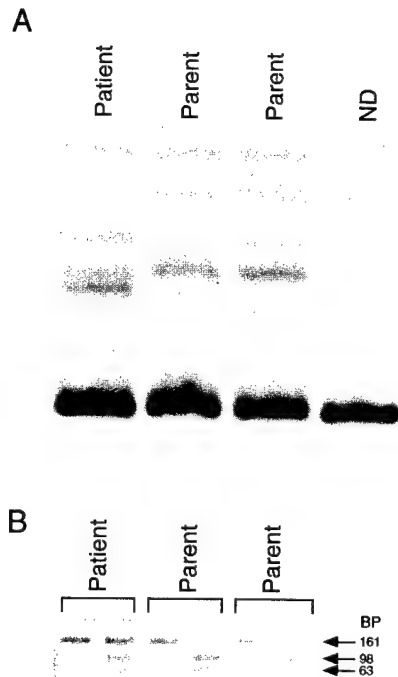


Figure 1 Alterations in exon 6 in patient 16221, relative to the patient's unaffected parents. *A*, SSCP analysis of exon 6. Both parents (mother and father) give a normal pattern, while the affected patient's sample gives several bands in addition to the normal bands. ND = non-denatured. *B*, Restriction-enzyme analysis. For each sample the first lane is the 161-bp exon 6 amplification product, and the second lane is the same product after digestion with the endonuclease *MspI*. Because the normal sequence contains an *MspI* recognition site (CCGG), both alleles in both parents (mother and father) are completely digested, to give products of 98 and 53 bp. The mutation carried by patient 16221 alters the recognition site to CCGT, so that the enzyme no longer digests the affected allele, while the patient's normal allele is digested completely.

identified had a similar familial distribution. Of the 12 individuals in whom no variation was found, 4 were the only affected member of their families, 5 had other affected relatives, and 3 had been lost to follow-up.

DNA Sequence Analysis

For each of the SSCP variants cited above, we determined the causative DNA sequence alteration in genomic DNA. The nature, location, and predicted protein effect of each mutation are listed in table 1. In all cases the affected individuals were heterozygous for the sequence change, as would be expected for an autosomal dominant disease. By far the most common alterations (19 of 20) are predicted to produce a shortened protein product. Splice acceptor mutations altering the highly conserved consensus sequence (agG) were seen in three individuals, and splice donor mutations altering the conserved sequence (Ggt) were seen in four individuals. Nonsense mutations producing premature stop codons were found in residues 57, 182, 262, 320, 341, 407, 463, and 466, with a single mutation at residue 527 being found in two unrelated individuals.

Single-base-pair deletions were seen in exons 10, 12, and 14. In all three cases the resulting frameshift led to a premature stop codon within 9-98 bp.

In only one case was a sequence alteration seen that would not be predicted to shorten the protein product substantially. In individual 15267 a 3-bp deletion in exon 3 removed a single phenylalanine residue at position 96 without causing a frameshift. This child affected with 8th and 5th cranial nerve tumors had a positive family history of NF2; however, DNA samples from other family members were not available.

Restriction-Digest Analysis

Because presymptomatic testing of family members is technically easier to accomplish with nonradioactive restriction-digest analysis than with SSCP or sequence analysis, the sequence changes listed in table 1 were examined for their ability to create or destroy a restriction-enzyme recognition site relative to the normal sequence. In seven cases the DNA sequence alteration seen in one allele would be predicted to create or destroy a restriction-enzyme recognition site (table 2). In each case these changes confirmed the sequence analysis in the individuals and, when available, their families. A typical example of this analysis for a sporadically affected patient and the patient's unaffected parents is shown in figure 1*B*.

Segregation of Alterations in NF2 Kindreds

In five cases (6272, 5434, 16039, 5095, and 13940) DNA samples from other family members were available to confirm segregation of altered alleles within the pedigree. For example, multiple affected members of two generations in the family of patient 13940 showed identical SSCP alterations of exon 12, while all unaffected individuals showed normal patterns (fig. 2). Single affected siblings of patients

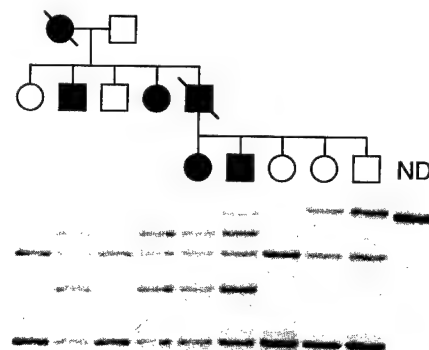


Figure 2 SSCP analysis of exon 12 in selected members of two generations of a large kindred segregating NF2. DNA samples from unaffected members of the pedigree produce the normal pattern, which includes several faint background bands. Samples from affected members of the pedigree consistently produce the same altered pattern. ND = nondenatured.

Table 1**NF2 Gene Mutations in Affected Unrelated NF2 Patients**

Patient	Exon	Sequence Change ^a	Codon Change ^b	Consequence
2815	8	784 C to T	Arg 262 X	Nonsense
4920	12	1176/1177 del 1 bp (G)	Glu 392 fs to 425 X	Frameshift
4974	12	1340/1341 +1 ins 1 bp (t)		Splice donor site
5095	11	1021C to T	Arg 341 X	Nonsense
5434	15	1579 G to T	Glu 527 X	Nonsense
6272	14	1499 del 1 bp (T)	Leu 500 fs to 514 X	Frameshift
7861	10	958 C to T	Gln 320 X	Nonsense
13158	2	169 C to T	Arg 57X	Nonsense
13940	12	1123-2 a to t		Splice acceptor site
14817	13	1396 C to T	Arg 466X	Nonsense
15267	3	285/288 to 287/290 del 3 bp (CTT)		del Phe 96
15572	15	1737+ 1 g to c		Splice donor site
16039	10	999 +1 g to a		Splice donor site
16097	2	240+1 g to c		Splice donor site
16209	5	448-2 a to g		Splice acceptor site
16212	5	448-2 a to t		Splice acceptor site
16215	10	997del 1 bp (C)	Gln 333 fs to 345 X	Frameshift
16221	6	544 G to T	Glu 182 X	Nonsense
16773	15	1579 G to T	Glu 527 X	Nonsense
16885	13	1387 G to T	Glu 463 X	Nonsense
NG1	12	1219 C to T	Gln 407 X	Nonsense

^a Numbering of bases showing alteration is given relative to the cDNA sequence, with the initiator ATG from Trofatter et al. (1993) beginning at base 1 (GenBank file L11353). All coding sequence bases are given in uppercase letters. When the alteration affects intronic sequence, it is presented in lowercase letters and numbered as “-” (5' intron) or “+” (3' intron) the requisite number of bases from the first or last base of the exon, respectively. For deletions, the span of deleted bases (numbered as above) is given, followed by the deletion size (“del”). For deletions of <5 bp, the deleted bases are also named. Where the start position of the deletion cannot be determined because of base-pair repetition, the alternative ranges of bases deleted are shown. Insertion is indicated by “ins” followed by the number of bases inserted and their identity.

^b The original amino acid and the position of the residues in the protein (numbered from the initiator Met as 1) are followed by the new amino acid (for missense mutation), by “X” (for nonsense mutation), or by “fs” (for frameshift), followed by the position of the next in-frame stop codon.

16039 and 6272 revealed, in exons 10 and 14, respectively, SSCP variations identical to those of their probands. A single affected sibling of patient 5095 revealed an altered-size fragment by *XhoI* digest of exon 11. The affected son of patient 5434 revealed an identical SSCP change in exon 15, while two clinically unaffected offspring were negative.

In cases 14817, 16209, 16212, 16215, 16221, and 13158 the affected individual was felt to have a new mutation to the disease; in each case, DNA from both unaffected parents did not show either the SSCP variant or, in the cases of 16209, 16212, and 16221, the restriction-enzyme-site variant (fig. 1A and B).

Table 2**Restriction-Enzyme Alterations in Affected Individuals**

Individual	Enzyme	Effect	Family Studies
4974	<i>HphI</i>	Site destroyed	None available
5095	<i>XhoI</i>	Site destroyed	Affected sibling
15572	<i>RsaI</i>	Site destroyed	None available
16097	<i>HindIII</i>	Site created	None available
16209	<i>MspI</i>	Site created	Parents negative (sporadic)
16212	<i>BsrI</i>	Site destroyed	Parents negative (sporadic)
16221	<i>MspI</i>	Site destroyed	Parents negative (sporadic)

Discussion

This paper describes our ongoing efforts to identify germ-line alterations of the NF2 gene in NF2-affected individuals. Through the use of SSCP analysis on blood lymphoblast DNA, we have characterized 20 alterations in 21 of 33 individuals studied. The majority—19 of 20—of these events would presumably result in a gross truncation of the predicted protein product, because of frameshift, creation of a stop codon, or interference with normal splicing. In a single case unique in its predicted protein-product outcome, a 3-bp deletion removes only a phenylalanine in exon 3 while preserving the reading frame. In

one case, two unrelated individuals carried the same mutation. All mutations identified segregated with the affected state when other family members were available for testing. Similar alterations were found both in isolated cases of NF2 and in individuals who were part of large kindreds segregating the disease. As in previous studies (Rubio et al. 1993; Jacoby et al. 1994), we have found a very low rate of benign polymorphism both in NF2-affected individuals and in individuals with sporadic tumors, by using these assays with over 300 chromosomes studied to date.

Our results confirm and expand the work of others who have examined germ-line mutation in NF2 patients. Jacoby et al. (1994), in a survey of eight patients, identified five germ-line mutations (two of which are also described in the present paper); all events would be predicted to produce gross truncation of the protein product. In the Jacoby et al. survey, two additional patients, who did not meet clinical criteria for NF2, were found to have germ-line mutations—one a missense mutation in exon 12 and one a possible acceptor branch-site mutation. Rouleau et al. (1993), in a survey of 90 patients, identified 15 events; however, only 6 of 17 exons were scanned. In a subsequent study scanning 10 exons in 12 tumors from NF2 patients, this group defined four additional inactivating events (Twist et al. 1994). In these two reports, 95% (18/19) of the changes—including 7 nonsense mutations, 6 frameshift mutations, and 5 splice-site-recognition mutations—would be predicted to grossly truncate the protein product; only one mutation would be predicted to substitute an amino acid (leucine to proline in exon 11). In these surveys, several apparently unrelated individuals carried the same nonsense mutations. We have found three individuals (patients 2815, 5095, and 13158) who carry mutations identical to those reported in these previous studies; to our knowledge, these patients are unrelated. A summary of the known germ-line mutations (excluding large deletions) in the NF2 gene is shown in figure 3. Although this summary demonstrates the presence of inactivating mutations at many different sites in the NF2 gene, it cannot be used to assess the relative frequency of mutation in each exon, since the studies by Rouleau et al. (1993) and Twist et al. (1994) did not scan the entire gene.

The results of the present survey of germ-line mutations stand in contrast to somatic mutations detected in this gene (see fig. 3). In the largest comprehensive study to date, 38 sporadic and NF2-derived schwannomas were scanned to identify 32 mutations (Jacoby et al. 1994). Although 90% (30 of 32) of the identified mutations produced gross truncation of the predicted protein product, the majority (20 of 30) of these events involved deletions of 1–61 bp, as compared with three deletions in the 20 germ-line events described here. Similar results were seen in three other studies of mutations in schwannomas. Sainz et al. (1993) characterized 11 mutations from 10 sporadic or NF2-de-

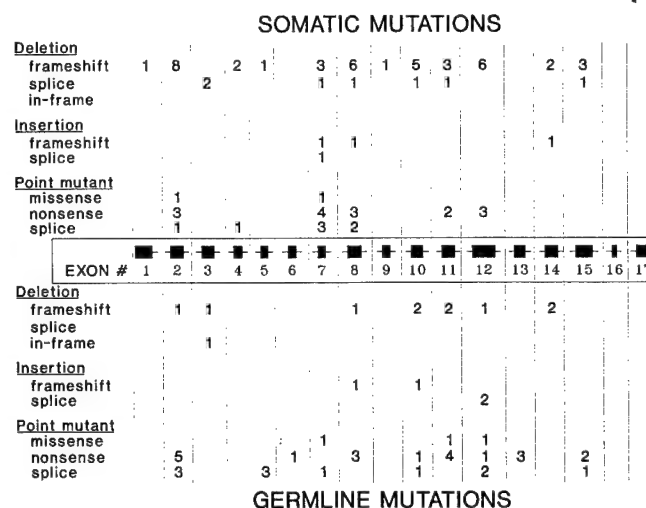


Figure 3 Summary of reported NF2 gene mutations. A schematic diagram is presented, illustrating the 17 exons of the NF2 gene. The number of independent mutations that alter each exon, as reported by MacCollin et al. (1993), Rouleau et al. (1993), Rubio et al. (1993), Trofatter et al. (1993), Irving et al. (1994), Jacoby et al. (1994), Rutledge et al. (1994), Twist et al. (1994), and the present study, are shown; they are divided, on the basis of their nature and predicted effect, into the categories listed on the left. Fifteen gene transcript mutations are presented in the report by Bianchi et al. (1994), but they are not included in this table, because splice mutation could not be distinguished from deletion in that report. Somatic changes are summarized above the gene, and germ-line alterations are displayed below the gene. Two germ-line mutations (one insertion in exon 10 and one deletion in exon 11) and two somatic splice mutations (both of which are deletions in exon 8) involve complex replacement of unequal numbers of base pairs. One somatic splice mutation in exon 8 lies in the coding sequence and also causes a potential missense event (GAG:gta to GAT:gta).

rived schwannomas; 9 of 11 were found to carry deletions of 1–136 bp. Twist et al. identified eight somatic alterations in 61 schwannomas; six of these eight involved deletion of 1–49 bp. Irving et al. (1994) identified 14 mutations in 95 schwannomas; 10 of 14 involved deletion. Interestingly, in a survey of tumor types not commonly associated with NF2, NF2 gene-transcript deletions were found in human breast adenocarcinoma, skin melanoma, and metastasis of skin melanoma (Bianchi et al. 1994). Most recently, Rutledge et al. (1994) have identified inactivating somatic mutation in meningioma specimens.

Although, thus far, the number of missense events found to affect merlin is low (a total of five; MacCollin et al. 1993; Rouleau et al. 1993; Jacoby et al. 1994; present study), these events may be important in elucidating the function of the merlin protein and its domains. Like the missense mutation previously described in a large NF2 pedigree (MacCollin et al. 1993), the phenylalanine found deleted in patient 15267 lies in a region highly conserved within the closely related members of this family of cytoskeletal associated proteins, including moesin, ezrin, and radixin. This finding further supports the notion that this

highly conserved domain is critical in the functioning of merlin as a tumor suppressor. Additional scanning of large numbers of patients should further define the critical residues in this domain and shed light on possible molecular interactions. The results described in the present report will also be the first step toward defining a potential genotype-phenotype relationship in NF2. Although detailed clinical data were not collected on the individuals studied in this work, to achieve these goals we are now studying a much larger and more clinically defined cohort of individuals.

As in previous studies, alterations were not identified in 100% of NF2 patients studied. Since linkage analysis in multiple families has failed to reveal heterogeneity (Narod et al. 1992), the likelihood that the NF2 phenotype may be due, in some cases, to a defect in a separate gene is low. Perhaps more likely is the possibility of alterations 5' or 3' to the translated region that affect transcription or translation of the NF2 message. Especially provocative is the finding that the first 100 bases of the 3' UTR show 70% identity in the mouse (Haase et al. 1994). In the present work, we have screened 60 bp of 5' UTR and 98 bp of 3' UTR, without finding alterations; further screening will involve larger segments of these regions. We may also have missed mutations in patients with deletions ranging from small events involving a single primer to large events removing the entire gene, which would interfere with the exon assays themselves. In our experience, alterations large enough to be detected by Southern blot analysis are rare (M. MacCollin, unpublished data) but do occur. Finally, it is clear that SSCP is not 100% sensitive and that some single-base-pair alterations may have been missed. Further studies are underway to compare the efficacy of alternate approaches to screening.

Perhaps the most immediate result of this effort is the ability to provide presymptomatic screening to at-risk family members, improving diagnostic certainty and reducing the need for costly radiographic and audiologic screening. For many family members this testing will serve to supplement an already ongoing screening program and should pose minimal additional psychological burdens. Testing of children at risk is a more problematic area, as it is unclear at this point if childhood detection will improve final outcome. In all cases both pre- and posttest genetic counseling will be essential for both affected and unaffected family members. With further studies we hope to both clarify the role of presymptomatic screening and improve clinical care for persons at risk for this disease.

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Analysis of the Neurofibromatosis 2 Gene in Human Ependymomas and Astrocytomas¹

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Abstract

Ependymomas and astrocytomas commonly have allelic losses of chromosome 22q, which suggests the presence of a glioma tumor suppressor gene on 22q. A candidate tumor suppressor gene on 22q is the neurofibromatosis 2 (*NF2*) gene since *NF2* patients have an increased susceptibility to ependymomas and astrocytomas. Using single strand conformation polymorphism analysis and direct DNA sequencing, we screened 8 ependymomas and 30 fibrillary astrocytomas from non-*NF2* patients for mutations in the coding sequence and portions of the 3' untranslated region of the *NF2* gene. Only one mutation was detected, a single base deletion in *NF2* exon 7 from a spinal ependymoma, which had also lost the wild-type allele. These results suggest that the *NF2* gene may be important in the formation of some ependymomas but the *NF2* gene is probably not the critical chromosome 22q tumor suppressor gene involved in astrocytoma tumorigenesis.

Introduction

Gliomas are the most common primary human brain tumors. These neoplasms are a diverse group that can be histopathologically divided into astrocytomas, oligodendrogliomas, and ependymomas (1). Molecular genetic analyses have identified patterns of allelic chromosomal loss in these tumors, suggesting a role for tumor suppressor genes in glioma tumorigenesis. In astrocytomas, frequent allelic loss has been noted for chromosomes 9p, 10q, 13q, 17p, 19q, and 22q (2). Oligodendrogliomas and ependymomas have been studied less extensively, but allelic loss of chromosome 19q is common in oligodendroglial tumors (3), and loss of chromosome 22q is frequent in ependymomas (4, 5). With the exception of the *p53* gene on chromosome 17p (6), however, distinct glioma tumor suppressor genes have not been implicated on these chromosomes.

A candidate glioma tumor suppressor gene on chromosome 22q is the recently cloned neurofibromatosis 2 (*NF2*) gene (7). *NF2* is an autosomal dominant syndrome in which patients develop bilateral vestibular schwannomas (acoustic neuromas), schwannomas in other sites, and multiple meningiomas. In addition, *NF2* patients have a higher incidence of gliomas, particularly ependymomas and, to a lesser extent, astrocytomas (8). We therefore evaluated the *NF2* gene in 8 sporadic ependymomas and 30 sporadic astrocytomas to determine whether the *NF2* gene is a glioma tumor suppressor gene on chromosome 22q.

Materials and Methods

All tumors were classified by a neuropathologist according to the WHO³ criteria (1). Of the eight ependymomas, four were WHO grade II and four had features of anaplastic ependymoma, WHO grade III; none of the tumors was of the myxopapillary type. Five of the ependymomas were fourth ventricular tumors; two were intramedullary spinal cord lesions; and one was located in the frontal lobe. All of the 30 astrocytic tumors were diffuse, fibrillary astrocytomas from the cerebral hemispheres of adult patients; two were WHO grade II; eight were anaplastic astrocytoma, WHO grade III; and 20 were glioblastoma multiforme, WHO grade IV. None of the patients had clinical or radiological evidence of *NF2*. DNA was extracted from fresh tumor (all astrocytomas and four ependymomas) and blood specimens according to standard phenol-chloroform procedures. DNA was extracted from formalin-fixed paraffin-embedded tissues of four ependymomas using a published protocol (9).

SSCP was performed as described (6) using published polymerase chain reaction conditions and oligonucleotides.⁴ All 17 published exons of the *NF2* gene and portions of the 3' untranslated region were screened. Amplification products larger than 200 base pairs were cleaved with appropriate restriction enzymes to yield fragments less than 200 base pairs in size. The amplification products were separated on 6-8% nondenaturing polyacrylamide gels with 10% glycerol overnight at 3-5 watts. Assays included positive control DNA from *NF2* patients or schwannomas with known *NF2* mutations.⁴ Cases with mobility shifts on SSCP were directly sequenced with Vent_R (exo⁻) DNA polymerase and the CircumVent Thermal Cycle Sequencing kit (New England BioLabs, Beverly, MA) using the SSCP or internal primers.

Results and Discussion

SSCP screening of the entire coding sequence of the *NF2* gene in 8 ependymomas and 30 fibrillary astrocytic tumors revealed a single migration shift which occurred in exon 7 of an ependymoma (Fig. 1, Lane 5). Only aberrantly migrating bands were noted in the tumor, implying loss of the remaining wild-type allele. DNA sequencing of this exon revealed a deletion of a single thymidine nucleotide (base 840) in codon 207 (Fig. 2, left). Only faint bands were present from the wild-type sequence, again implying loss of the remaining wild-type allele. The frameshift mutation resulted in a stop in codon 208, thus leading to a severely truncated protein product. To date, similar frameshift and nonsense mutations have been the most common types of mutations detected in the *NF2* gene in schwannomas and in *NF2* patients.⁴ The combination of mutation of one *NF2* allele and chromosomal loss of the second 22q allele thus fulfills the classic paradigm of a recessively-acting tumor suppressor gene.

The tumor with an *NF2* gene mutation was a recurrent, intramedullary cervical ependymoma, WHO grade II, from a 45-year-old man without family history or stigmata of *NF2*. Sequencing of the patient's constitutional DNA revealed the wild-type sequence (Fig. 2, right),

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³ The abbreviations used are: WHO, World Health Organization; SSCP, single strand conformation polymorphism analysis.

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confirming the somatic nature of the mutation. Histological examination of the original tumor revealed no atypical or anaplastic features. Although formalin-fixed, paraffin-embedded tissue was available from the first tumor, adequate DNA could not be extracted.

Molecular genetic and cytogenetic studies have demonstrated that chromosome 22q loss is common in ependymomas (4, 5, 10). While such analyses have suggested that chromosome 22q harbors an ependymoma tumor suppressor gene, they have not narrowed down the location of this putative tumor suppressor gene. Our data implicate the *NF2* gene as the target of 22q allelic loss in at least some ependymomas. It is tempting to speculate that *NF2* mutations may be particular to intramedullary spinal ependymomas since these are the types of ependymomas characteristically associated with NF2 (11) and we did not detect *NF2* mutations in any of the six intracranial ependymomas. Alternatively, the lack of mutations in the remainder of cases may imply that the presence of a second chromosome 22q ependymoma tumor suppressor gene or that *NF2* mutations may occur in nonexonic portions of the gene, such as in promoters or introns, or in additional, alternatively spliced exons. Our recent screening of the *NF2* gene in NF2 patients and schwannomas has revealed a considerable number (approximately 50%) of cases without detectable mutations in the

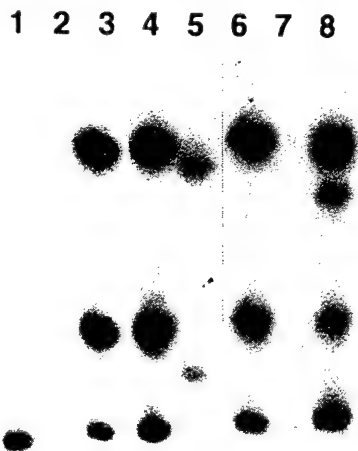


Fig. 1. SSCP analysis of *NF2* exon 7 in four ependymomas (Lanes 3-6) showing migration shift and loss of the wild-type bands in Lane 5. Lane 1, nondenatured DNA; Lanes 2 and 7, "no DNA" controls; Lane 8, positive control from a NF2 patient. Note only aberrantly migrating mutant allele in tumor (Lane 5) whereas constitutional DNA from NF2 patient has both mutant and wild-type alleles (Lane 8).

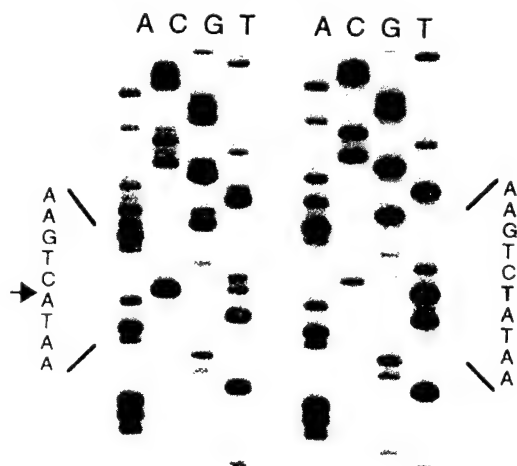


Fig. 2. Sequence analysis reveals loss of a thymidine in *NF2* codon 207 in the ependymoma (left) compared to the normal sequence (right). Note some superimposed faint bands above the deletion site as a result of wild-type allele contamination.

1 2 3 4 5 6 7 8 9 10 11 12 13



Fig. 3. SSCP analysis of *NF2* exon 10 in a series of astrocytomas and ependymomas (Lanes 3-11). No migration shifts are noted except in the positive control from a NF2 patient (Lane 13). Lane 1, nondenatured DNA; Lane 2, "no DNA" control.

same regions assayed in the present study,^{4, 5} supporting the possibility that mutations may occur in other regions of the gene. Finally, the lack of mutations in the remainder of the ependymomas may be due to problems in the screening method since SSCP may not detect all point mutations and is not an adequate means of identifying larger genomic deletions. Such larger genomic deletions have already been noted in several NF2 patients (7).

None of the thirty astrocytomas had detectable mutations in the *NF2* gene (Fig. 3). These findings make it improbable that the *NF2* gene is the critical astrocytoma tumor suppressor gene on chromosome 22q, unless mutations occur exclusively in other regions of the gene. Three astrocytomas have been reported with loss of heterozygosity at *D22S171* on distal 22q but with maintenance of heterozygosity at the more proximal locus *D22S80* (12). Since *D22S80* is distal to *NF2*, these findings support our conclusion that the *NF2* gene is not the chromosome 22q astrocytoma tumor suppressor gene and that the putative gene lies distal to *NF2*. Further detailed deletion mapping of chromosome 22q in astrocytomas may help to narrow down the location of this distal culprit.

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Mutational Analysis of *CDKN2* (*MTS1*/p16^{ink4}) in Human Breast Carcinomas¹

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Abstract

The *CDKN2* gene that encodes the cell cycle regulatory protein cyclin-dependent kinase-4 inhibitor (p16) has recently been mapped to chromosome 9p21. Frequent homozygous deletions of this gene have been documented in cell lines derived from different types of tumors, including breast tumors, suggesting that *CDKN2* is a tumor suppressor gene involved in a wide variety of human cancers. To determine the frequency of *CDKN2* mutations in breast carcinomas, we screened 37 primary tumors and 5 established breast tumor cell lines by single-strand conformation polymorphism analysis. In addition, Southern blot analysis was performed on a set of five primary breast carcinoma samples and five breast tumor cell lines. Two of the five tumor cell lines revealed a homozygous deletion of the *CDKN2* gene, but no mutations were observed in any of the primary breast carcinomas. These results suggest that the mutation of the *CDKN2* gene may not be a critical genetic change in the formation of primary breast carcinoma.

Introduction

A new putative tumor suppressor gene with a nomenclature *CDKN2*³ (1, 2) has been mapped to chromosome 9p21, a region in which molecular, genetic, and cytogenetic abnormalities have been characterized in several malignant tumors and cell lines including melanomas (3, 4), gliomas (5), lung cancers (6), and leukemia (7). The *CDKN2* gene encodes the previously identified protein p16, a cell cycle regulatory protein that binds to CDK4⁴ and inhibits the catalytic activity of the CDK4/cyclin D enzymes (1, 2, 8). The CDK4-cyclin D complexes control the cell passage through the G₁ phase of the cell cycle by phosphorylation of the retinoblastoma protein (9). However, interaction of CDK4 and p16 inhibits CDK4 function (8). The biochemical properties of p16 suggest that it could act as a negative regulator of proliferation of normal cells. This view has been supported by recent studies which demonstrated frequent homozygous deletions of the *CDKN2* gene in cell lines derived from different tumor types including glioma (71–87%), breast cancer (60%), melanoma (57–61%), and leukemia (25–65%; Refs. 1 and 2). These results, generated by screening established tumor cell lines, imply that the *CDKN2* gene may function as a tumor suppressor, and mutations in *CDKN2* may be involved in tumor formation in a wide range of human cancers.

Karyotyping of sporadic primary breast carcinomas has revealed breakpoints and deletions involving many chromosomes, including 9p

(10, 11). However, loss of heterozygosity studies in breast carcinomas do not report allele loss of chromosome 9p (12, 13). Although homozygous deletions of *CDKN2* were detected in 60% of established breast cancer cell lines (1), the involvement of this gene in primary breast carcinomas remains to be established. To determine whether the *CDKN2* gene plays a role in tumorigenesis of primary breast carcinomas, we examined the entire coding region of this gene for mutations in 37 primary sporadic breast carcinomas (14 lobular carcinomas and 23 ductal carcinomas) and 5 established breast tumor cell lines.

Materials and Methods

Cases diagnosed as primary breast carcinoma were obtained by reviewing the pathology files of Massachusetts General Hospital spanning the years 1991 to 1993. All tumors were classified according to the WHO classification. Five breast tumor cell lines were generously provided by Dr. Stephen Friend. (Massachusetts General Hospital, Boston, MA). Genomic DNA was extracted from frozen tumor tissues and cell lines using standard procedures. DNA was extracted from formalin-fixed paraffin-embedded tumor tissues using a published protocol (14).

SSCP analysis was carried out in the presence of 5% dimethyl sulfoxide using *CDKN2*- and *MTS2*-specific intronic flanking oligonucleotides. PCR conditions and the sequences of the oligonucleotides are published elsewhere (15). A cosmid containing *CDKN2* was included in the SSCP assays as a positive control. The amplification products were separated on both 6 and 8% nondenaturing polyacrylamide gels containing 8% glycerol at 6–8 W for 14–16 h. PCR products were either directly sequenced as described earlier (16) or cloned into TA cloning vector (PCR II; Invitrogen), and at least six clones for each product were sequenced by double-stranded sequencing. For Southern blot analyses, DNAs from randomly chosen frozen tumor tissues and cell lines were digested with *EcoRI* and *HindIII* and hybridized with *CDKN2*-specific complementary DNA probes generated by RT-PCR amplification. The same blots were also hybridized with a single copy exon-specific probe obtained from the neurofibromatosis 2 gene as a control. The blotting and the hybridizations were performed as described previously (17).

Results and Discussion

The coding region of *CDKN2* spans three exons (1, 2), all of which were screened using at least two different sets of SSCP conditions. The analysis was carried out on DNA from a total of 37 primary breast carcinomas that included 23 ductal carcinomas as frozen tumor material and 14 infiltrating lobular carcinomas as formalin-fixed, paraffin-embedded blocks. Samples with high tumor cell concentration (>70%) were identified to minimize nontumor cell contamination. In addition, the analysis included DNA obtained from five established breast tumor cell lines. PCR primers were designed to amplify approximately 200–250-base pair fragments or larger fragments that were subsequently digested with appropriate restriction enzymes to yield fragments of approximately 200–250 base pairs (15).

SSCP screening of the entire coding sequence of *CDKN2* revealed no tumor-specific migration shift in any of the primary breast carcinomas analyzed. However, two of the five established breast tumor cell lines failed to amplify all three exons of the *CDKN2* gene,

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³ *MTS1* (multiple tumor suppressor 1 gene) has been designated as *CDKN2* by the HUGO Nomenclature Committee.

⁴ The abbreviations used are: CDK4, cyclin-dependent kinase-4; SSCP, single-strand conformation polymorphism; *MTS2*, multiple tumor suppressor 2; RT-PCR, reverse transcription-polymerase chain reaction.

suggesting a homozygous deletion of *CDKN2* in these two cell lines. The other three cell lines did not reveal migration shifts by SSCP. In addition, the SSCP analysis revealed two polymorphisms in *CDKN2*, one in exon 2 and one in the 3' untranslated region. The exon 2 variation, GCG to ACG, substituted threonine for alanine at codon 140. This change was observed in one primary tumor as well as in DNA obtained from a normal control individual (data not shown). SSCP analysis of exon 3, which included the 3' untranslated region, revealed migration shifts in a total of seven samples, and representative samples are shown in Fig. 1. All seven samples exhibited the same variation (Fig. 1, Lanes 2 and 9), and in addition, Fig. 1, Lane 9 showed missing bands. Sequencing of these variations revealed a C to G polymorphism in base 494 of the 3' untranslated region (8). Six samples were heterozygous for this variation, and the one sample that showed missing bands (Fig. 1, Lane 9) was homozygous. These two polymorphisms are described in detail elsewhere (15). SSCP screening was also performed for exon 2 of the *MTS2* gene (1), and no migration shifts were observed in any of the samples. It should be noted, however, that the two breast tumor cell lines that exhibited homozygous deletion for the entire *CDKN2* gene had intact *MTS2* exon 2.

To ensure that we did not overlook any gross rearrangements of *CDKN2* in these tumors, we also performed Southern blot analysis on a set of DNAs from five primary breast carcinomas and five tumor cell lines digested with *EcoRI* and *HindIII* (Fig. 2). The *CDKN2* probe was obtained by RT-PCR amplification of the human p16 complementary DNA spanning 257 to 914 base pairs (8), which identified fragments of about 4.2 and 9.3 kilobases with *EcoRI* and *HindIII*, respectively. Southern blot analysis confirmed the homozygous deletion of the *CDKN2* gene in the two tumor cell lines [Fig. 2, Lanes 6 (Hs 578T) and 9 (MCF 7)] but did not reveal any other major rearrangements in other samples. Some minor band shifts were observed in a number of lanes in these blots, and to clarify this variation,

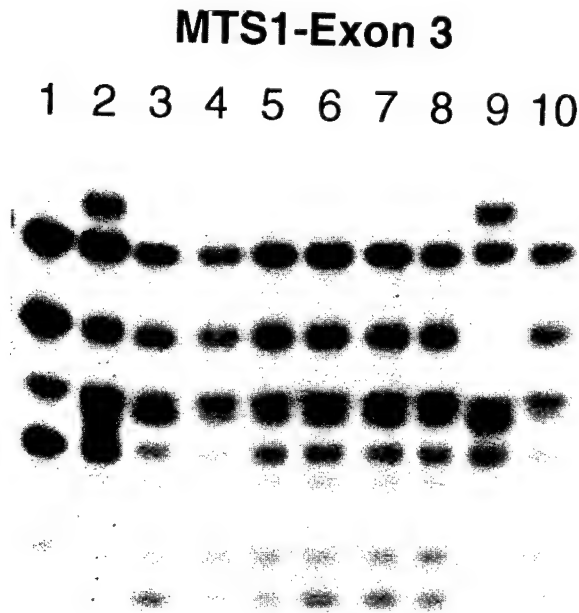
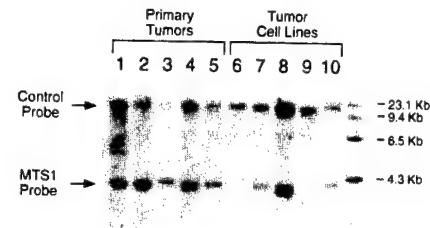


Fig. 1. SSCP analysis of exon 3 of the *CDKN2* gene in representative primary breast carcinoma samples. Sample 2 reveals the migration shift that was also identified in five other samples. Sample 9, in addition to this shifted fragment, also revealed other variations.

A. *EcoRI*



B. *HindIII*

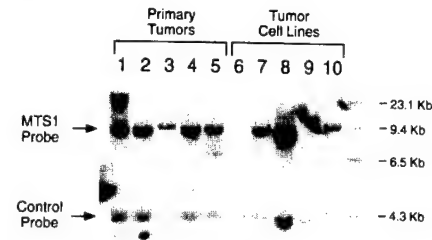


Fig. 2. Southern blot analysis of genomic DNA from five breast carcinomas (Lanes 1-5) and five breast tumor cell lines (Lanes 6-10). DNA (5-10 μ g) were digested with *EcoRI* (A) and *HindIII* (B), size fractionated on a 1% agarose gel, transferred, and hybridized to a RT-PCR-amplified *CDKN2* probe. The same blots were rehybridized to a control single copy probe from the neurofibromatosis 2 gene. The breast cancer cell lines included Hs 578 T, SK-BR-3, Du4475, MCF 7, and ZR-75-30 (Lanes 6-10, respectively).

we reprobbed these blots with a control single copy probe from the neurofibromatosis 2 gene. Similar band shifts were noted, suggesting a gel/sample artifact. The two cell lines with *CDKN2* deletion, however, showed intact fragments for the control probe, confirming their integrity of the genomic DNA (Fig. 2). In addition, we performed multiplex PCR reactions of exons 1 and 2 of the *CDKN2* gene in combination with a locus distal to *CDKN2* on chromosome 9p in all tumors and cell lines and found no variation in the tumors and detected the homozygous deletion of *CDKN2* in the same two cell lines (data not shown).

Using SSCP screening and Southern blot analyses, we were not able to find any alterations in the *CDKN2* gene in a total of 37 primary breast carcinomas, implying that mutation of the *CDKN2* gene may not be a critical genetic change in the formation of primary breast carcinoma. However, similar to a previous study (1), homozygous deletion of the *CDKN2* gene was detected in two of the five established breast tumor cell lines. Parallel to our observations, recent studies performed on other primary tumors with known allelic loss of 9p, such as astrocytomas (15), bladder tumors (18, 19) lung, head and neck, brain, and kidney (19) have reported *CDKN2* mutations only in a small fraction of primary tumors. In contrast, Mori *et al.* (20) has detected somatic mutations in 14 of 27 squamous cell carcinomas. Therefore, *CDKN2* may play a role in certain tumor types; however, further systematic studies of *CDKN2* in other primary tumors and cell lines are needed to clarify the relationship between *in vivo* and *in vitro* alteration of *CDKN2*.

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Neurofibromatosis 2 Gene in Human Colorectal Cancer

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ABSTRACT: Colon cancers commonly have allelic losses of chromosome 22q, which suggests the presence of a tumor suppressor gene on 22q. The candidate tumor suppressor gene on 22q is the neurofibromatosis 2 (NF2) gene. Using single strand conformation polymorphism (SSCP) analysis, we screened 24 pairs of colorectal cancer and adjacent normal mucosa, as well as 10 colon cancer cell lines from non-NF2 patients, for mutations in the coding sequence of the NF2 gene. Two SSCP variants, one in exon 14 and another one in exon 16, were detected in two of the sporadic colorectal cancers, but not in adjacent normal mucosa samples. Sequencing of these variants in one tumor detected an A-to-G transition in bp 1459 of the NF2 cDNA, resulting in the change of Ile to Val at codon 487 of merlin, the NF2 protein product. The other tumor showed a 2-bp (CT) deletion in the intronic sequence of the alternatively spliced exon 16. These results suggest that the NF2 gene is probably involved in some colorectal tumors, but is not the critical chromosome 22q tumor suppressor gene involved in colon tumorigenesis.

INTRODUCTION

Sporadic colon cancers are among the most common primary human tumors. Molecular genetic analyses have identified patterns of allelic chromosomal loss in these tumors, suggesting a role for tumor suppressor genes in colon tumorigenesis. In colon cancers, frequent allelic loss has been identified in chromosomes 5q (30%), 8p (40%), 17p (75–80%), 18q (80%), and 22q (20–30%) [1]. Indeed, much has been published on the tumor suppressor genes APC, p53, and DCC, which have been localized to chromosomes 5q, 17p, and 18q, respectively. Genetic alterations in these genes are detectable in the majority of sporadic colon cancers [2–5].

A candidate tumor suppressor gene on chromosome 22q is the recently cloned neurofibromatosis 2 gene [6, 7]. The NF2 gene encodes the protein merlin, a novel member of a family of proteins that are thought to link membrane proteins with the cytoskeleton. NF2 is an autosomal dominant syndrome in which patients develop bilateral vestibular schwannomas, schwannomas in other sites, and multiple meningiomas [8]. Colorectal cancer is not part of the NF2 syndrome. However, given the frequently reported allelic loss of chromosome 22q in sporadic colorectal cancer, we evaluated the mutations in the NF2 gene in 24 pairs of sporadic colorectal tumors with adjacent normal mucosa, as well as 10 colon cancer cell lines, to determine whether the NF2 gene

is a tumor suppressor gene involved in colonic tumorigenesis.

MATERIALS AND METHODS

All tumors were classified according to the Dukes classification. The 24 colon tumors were divided equally based on the degree of differentiation in Dukes stages A, B, C, and D. None of the patients had clinical or radiologic evidence of NF2. In addition, 10 colon cancer cell lines obtained from the American Tissue Type Collection (ATCC) were analyzed. DNA was extracted from fresh tumor obtained at the time of surgical resection. Adjacent normal mucosa was obtained concurrently and served as control. DNA was extracted from tissue and cell lines according to standard phenol-chloroform procedures.

Single strand conformation polymorphism (SSCP) analysis was performed as described using published polymerase-chain reaction conditions and oligonucleotides [9]. The amplification products were separated on 6–8% nondenaturing polyacrylamide gels containing 8% glycerol at 6–8 W for 14–16 hours. Cases with mobility shifts on SSCP were sequenced directly as described earlier [9] or cloned into TA cloning vector (pCR II In vitrogen) and at least five clones were sequenced by double-stranded sequencing using Sequenase version 2.0 (US Biochemicals).

RESULTS AND DISCUSSION

The coding region of the NF2 cDNA spans at least 17 exons, with exon 16 alternatively spliced in both human and mouse [9–11]. All 17 exons of the NF2 gene were screened by SSCP for any sequence variations showing migration shifts. SSCP screening of the entire coding sequence of the NF2 gene in

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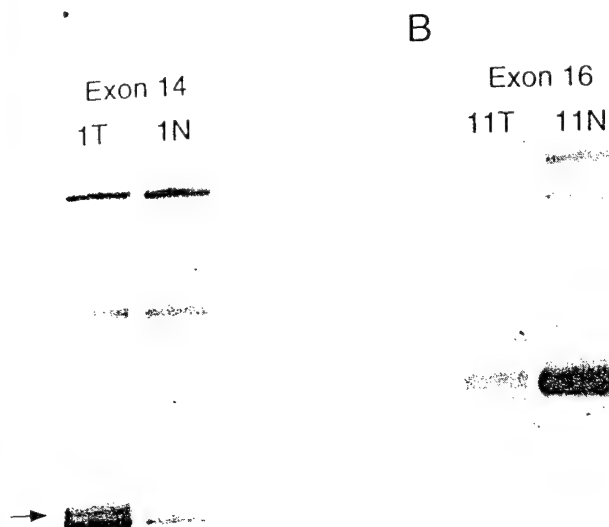


Figure 1 A) SSCP analysis of exon 14 in colon tumor 1T and the corresponding mucosa 1N. The arrow indicates the variation observed in 1T. B) SSCP analysis of exon 16 in colon tumor 11T and the mucosa 11N; the variation observed in 11T is clearly seen.

24 colorectal tumors with adjacent normal mucosa as well as 10 colon cancer cell lines revealed two different migration shifts in two different tumor samples that were not present in adjacent normal mucosa. No migration shifts were ob-

served in colon cancer cell lines. The tumor 1T revealed a SSCP variation in exon 14 and the tumor 11T showed a variation in exon 16. The corresponding normal mucosa samples 1N and 11N did not reveal these variations (Fig. 1). None of the other pairs analyzed revealed any variations. On sequencing the variants, we observed an A-to-G transition in tumor 1T that resulted in the substitution of Ile to Val at amino acid position 487 in exon 14. This mutation was seen in one allele. DNA sequencing of tumor 11T revealed a 2-bp deletion in one allele spanning + 19-20 in the splice donor site of intron 16. The corresponding mucosa samples 1N and 11N did not show these sequence changes (Fig. 2). Several independent PCR products were also cloned in TA vector and a number of clones were sequenced from both the tumor/mucosa pair samples. The Ile to Val change was observed in 9/27 clones for tumor 1T, but in 0/10 clones from 1N had this change. The 2-bp deletion in intron 16 was observed in 3/5 clones, for 11T, but in 0/5 in 11N. The absence of sequence variations in the adjacent mucosa samples, as well as in an extensive screening of a variety of other tumors and in NF2 patients, strongly suggest that the Ile to Val change is not a polymorphism. However, the Ile 487 to Val retains the hydrophobic nature of the amino acid side chain, the significance of which is unknown. The 2-bp deletion occurs in one of the three stretches of CT sequences in intron 16 at position + 19-20 in the splice donor site. To verify whether this mutation affects the splicing of exon 16 in this tumor we performed RT-PCR analysis on this pair of samples and four other pairs of samples, which did not reveal any se-

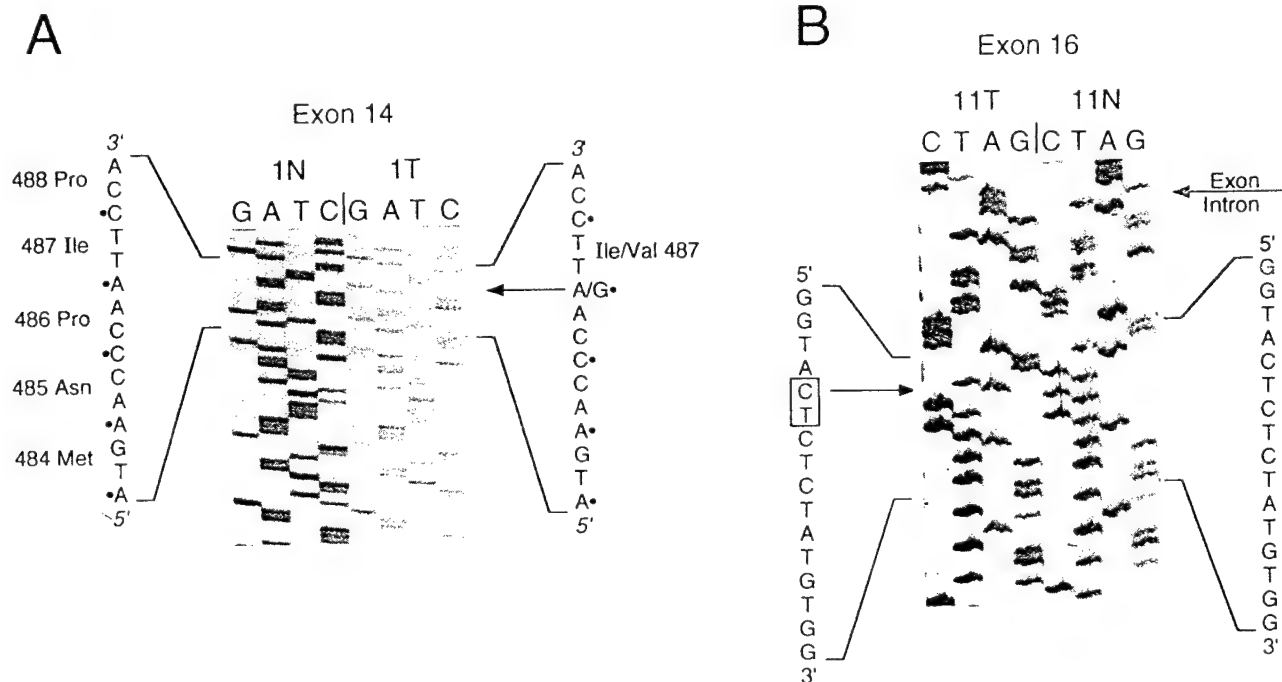


Figure 2 A) Double-stranded sequence analysis of cloned PCR products of 1N and 1T. The tumor sample 1T reveals an A-to-G transition change resulting in Ile 487 to Val in exon 14. B) Double-stranded sequence analysis of cloned PCR products of 11T and 11N. The tumor sample 11T revealed CT deletion which is shown in the box. The splice junction is indicated by the exon intron boundary.

quence changes. RT-PCR analysis showed the expression of isoforms with and without exon 16 in all the samples. It was difficult to quantitate the ratio of both isoforms in 11T in comparison to other tumor samples (data not shown).

Although the two changes reported here are clearly seen only in tumor samples, the significance of the mutations is not clear. Arakawa et al. has recently reported Ala 395 to Val and Ser 307 to Tyr in 2/44 colorectal cancers analyzed [12]. Further studies are necessary to evaluate whether these mutations alter the expression or function of *merlin* in these tumors. While molecular genetic and cytogenetic studies have demonstrated chromosome 22q loss in colorectal cancers, they have not narrowed the location of the putative tumor suppressor gene. Although NF2 gene is probably involved in some colon tumors, it may not be the critical tumor suppressor gene on chromosome 22q for these tumors unless mutations occur exclusively in other regions of the gene such as the promotor region or in additional alternatively spliced exons. NF2 mutations have been well documented in NF2-related tumors such as vestibular schwannomas and meningiomas [9, 13, 14]. However, they appear not to be critical in astrocytomas that also display frequent allelic loss of chromosome 22q [15]. These findings support the notion that there could be other tumor suppressor gene(s) on chromosome 22q that could play a role in other types of tumors harboring frequent 22q loss.

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The merlin tumor suppressor localizes preferentially in membrane ruffles

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Merlin is a tumor suppressor whose inactivation underlies the familial schwannomas and meningiomas of neurofibromatosis 2 and their sporadic counterparts. It bears striking similarity to the ERM proteins, ezrin, radixin and moesin, members of the protein 4.1 superfamily that link proteins in the cytoskeleton and the plasma membrane. We have generated polyclonal and monoclonal antibodies that detect merlin as an ~66 kD protein in many different cell types. Using indirect immunofluorescence we have for the first time visualized endogenous merlin and localized it to the motile regions, such as leading or ruffling edges, in human fibroblast and meningioma cells. Merlin co-localizes with F-actin in these motile regions but is not associated with stress fibers. Merlin does not localize to the same structures as either ezrin or moesin in human meningioma cells, suggesting a function distinct from these ERMs. Thus, merlin is associated with motile regions of the cell and its participation in these structures may be intimately involved in control of proliferation in Schwann cells and meningeal cells.

Keywords: merlin; neurofibromatosis 2; meningioma; vestibular schwannoma; ERM; motility

Introduction

We recently identified merlin (for moesin, ezrin, radixin-like protein), a novel member of the protein 4.1 superfamily, as the tumor suppressor associated with neurofibromatosis 2 (NF2) (Trofatter *et al.*, 1993), a genetic disorder typified by vestibular schwannomas (benign, slow growing tumors on the vestibular branch of the 8th cranial nerve) that occur bilaterally and by frequent meningiomas, spinal schwannomas and ependymomas. The same tumor types also occur as sporadic, solitary tumors in the general population due to somatic merlin inactivation. NF2 has a profound impact on its victims, as multiple slow-growing tumors gradually compress adjacent nerves, causing deafness, disrupted balance control, paralysis and early death. More recently, merlin mutations have been detected in non-NF2 tumors, particularly mesotheliomas, extending its potential range of tumor suppressor function (Bianchi *et al.*, 1995; Sekido *et al.*, 1995).

Proteins of the band 4.1 superfamily are defined by a

homologous domain of ~270 amino acids near the N-terminus (Arpin *et al.*, 1994; Luna and Hitt, 1992). Like the archetype, erythrocyte protein 4.1, three members of the family, ezrin, moesin and radixin (known collectively as ERM proteins) act as molecular links between the cytoskeleton and the plasma membrane. ERMs share 70–75% amino acid identity and each consist of a protein 4.1 domain followed by a long α -helical domain and a charged COOH-terminus. These proteins are located in actin-rich surface projections such as microvilli in cultured cells and intestinal epithelia (Bretscher, 1983), filopodia, membrane ruffles and lamellipodia in migrating cells (Birgbauer, 1991) as well as in neuronal growth cones (Birgbauer, 1991; Gonzalez-Agosti and Solomon, 1996; Goslin *et al.*, 1989), and cleavage furrows in dividing cells (Henry *et al.*, 1995; Sato *et al.*, 1991). ERMs interact with each other (Androeli *et al.*, 1994; Gary and Bretscher, 1993) and with actin (Pestonjamas *et al.*, 1995; Turunen *et al.*, 1994) and with the extracellular matrix receptor CD44 as a membrane target (Tsukita *et al.*, 1994).

Merlin is 45–47% identical to the ERM proteins and shares a common structural pattern except that it has two different charged carboxy-termini produced by alternative splicing (Bianchi *et al.*, 1994; Jacoby *et al.*, 1994). The similarity with the ERM proteins suggests that the tumor suppressor activity of merlin may involve a novel mechanism that includes a role for cytoskeleton-membrane interaction. Experiments performed with antisense oligonucleotides complementary to ERM sequences have indicated that these members may play a role in cell-cell and cell-substrate adhesion, probably through the regulation of actin filament-plasma membrane interactions (Takeuchi *et al.*, 1994). The strong similarity at the amino-terminus of merlin and the ERM proteins (63% identity) suggests that merlin may also associate with both membrane and cytoskeletal structures. However, the existence of two major merlin isoforms with alternative carboxy-termini dissimilar from those of ezrin, radixin, and moesin indicates that merlin probably plays a role distinct from the ERM proteins. When merlin's distinct function is disrupted, growth regulation can be lost and tumor formation results.

We have generated several polyclonal and monoclonal antibodies against different regions of this protein to explore its cellular localization. These antibodies detect a variably expressed ~66 kD protein in Western blot analysis of a variety of human cell lines. In immunocytochemical analysis, the monoclonal antibody 1C4 detects endogenous merlin which localizes preferentially in the motile regions of cells such as the leading or ruffling edges. In these regions, merlin co-localizes with F-actin, but the localization is

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distinct from those of ezrin and moesin in meningioma cells. These data support the view that while the structure of merlin may be similar to ERMs, its function is distinguishable. The presence of merlin in the motile regions of the cell suggests that accurate regulation of cell movement, shape or communication may be crucial to control Schwann cell and meningeal cell proliferation.

Results

Expression of merlin in different cell types

To study the normal biological function of the NF2 protein merlin and its role as a tumor suppressor, we have raised various polyclonal antibodies in rabbits using synthetic peptides (MP4, MP5, MP7) derived from regions of merlin not exhibiting similarity to the protein 4.1 family members, as well as bacterially expressed GST-MERN21 (N21). In addition, a chicken polyclonal antibody was raised using the GST-MERC26 as an antigen (H985) and a monoclonal antibody (1C4) that recognizes both isoforms of merlin was raised against the carboxy-terminus of the protein (see Materials and methods).

The detection of merlin in protein extracts of human fetal fibroblast cell line MRC-5 by all of our anti-merlin antibodies is shown in Figure 1a. These antibodies, with the exception of N21, detect a single specific band of ~66 kD not observed with the corresponding preimmune sera. The rabbit polyclonal antibody N21 which was raised against the amino-terminus (spanning amino acids 1-332) detects both merlin at ~66 kD and two larger bands at ~78 kD and ~82 kD. The larger bands detected by the N21

antibody co-migrate with ezrin and moesin but this antibody has not revealed a band co-migrating with radixin in any cell lysate tested thus far (data not shown).

The monoclonal antibody 1C4 was raised against the carboxy-terminus of merlin, which exhibits only weak similarity to the ERMs. To confirm the merlin specificity of 1C4, we performed Western blot analysis in parallel with specific ERM antibodies using MRC-5 protein extracts. The nitrocellulose membrane with the transferred protein was stained with Ponceau-S, each lane was divided into two strips and the alternate strips were probed with 1C4 and with a specific anti-peptide antibody against either ezrin, radixin, or moesin. Like the polyclonal antisera, 1C4 detects a distinct 66 kD protein (Figure 1b) migrating more rapidly than moesin, radixin, and ezrin. 1C4 also immunoprecipitates the 66 kD band from various human cell lysates (data not shown).

We have examined the expression of merlin in a variety of human cell lines by Western blot analyses with our antisera. Examples of these using the monoclonal 1C4 are shown in Figure 2. Merlin migrates as an ~66 kD in various human cell lines (Figures 2a and b) including adult fibroblasts, fetal fibroblasts (MRC-5), glioma cells (H238), HeLa cells as well as in two primary meningioma cell lines established from sporadic meningiomas without NF2 mutations (MN12 and MN27). The latter tumors belong to that subset of sporadic meningiomas thought to be due to genetic lesions in an as yet unidentified locus rather than the mutations in the NF2 gene (Ruttledge *et al.*, 1994; Wellenreuther *et al.*, 1995). Other meningioma cell lines from the tumors of NF2 patients with known mutations such as MN11 were included as negative controls (Figure 2b). 1C4 also

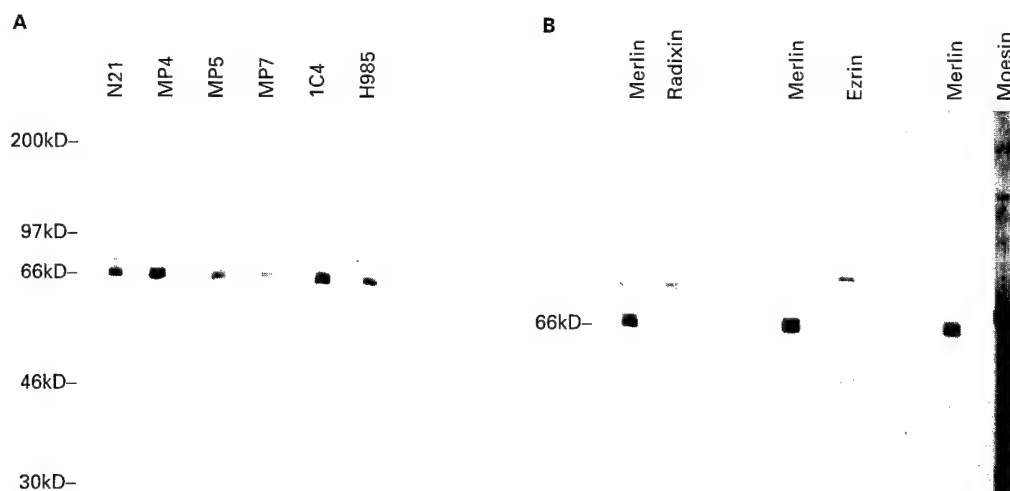


Figure 1 Western blot analysis with different anti merlin antibodies. (a) Human fetal fibroblast (MRC-5) cell lysate was fractionated on a 7.5% SDS-polyacrylamide gel, blotted on nitrocellulose and immunoprobed with various rabbit polyclonal antibodies (N21, MP4, MP5, MP7), mouse monoclonal antibody (1C4) and a chicken polyclonal antibody (H985). All the polyclonal antibodies were affinity purified. The single band (with the exception of N21) detected by the different antibodies has an apparent molecular mass of approximately 66 kD. None of the preimmune sera detect this band. (b) Monoclonal antibody 1C4 detects merlin but not the ERM proteins. Western blot analysis was performed with 1C4 in parallel with specific ERM polyclonal antibodies using protein extracts from MRC-5 cells. 1C4 detects a single distinct 66 kD protein migrating rapidly than moesin, radixin and ezrin

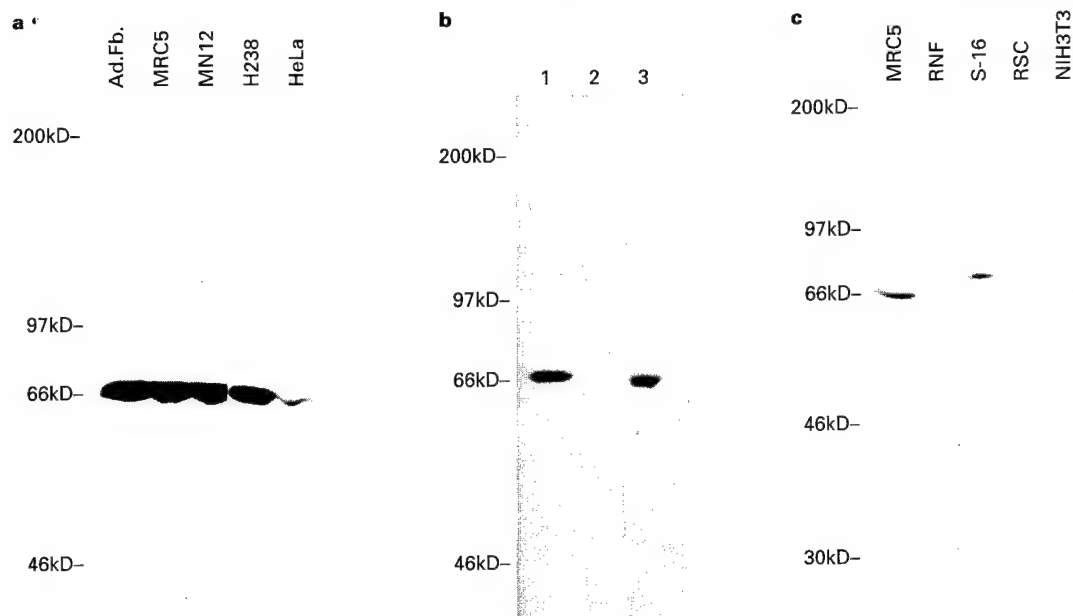


Figure 2 Analysis of expression of merlin by immunoblot using the mAb 1C4. Approximately 600 μ g of protein extract from various human cell lines and 800 μ g of protein extract from rodent cell lines were analysed on a 7.5% SDS-PAGE. (a) Includes human adult fibroblasts, fetal fibroblasts (MRC-5), primary meningioma cell line MN12, glioma cells (H238) and HeLa cells. (b) A panel of primary meningioma cell lines MN12 (lane 1) and MN27 (lane 3) without mutations in the *NF2* gene, and meningioma cell line MN11 (lane 2) as a negative control from the tumor of a NF2 patient with a known mutation. (c) Protein extracts from rodent cell lines include rat newborn fibroblast (RNF), rat Schwann cell line (S-16), primary rat Schwann cells (RSC) and NIH3T3. MRC-5 is included as a control. While 1C4 detects merlin as a 66 kD band in most of these cell lysates, extracts from RNF and S-16 reveal a higher migrating protein at approximately 72 kD

detects rodent merlin as an ~66 kD band in Western blot analysis of lysates from NIH3T3 cells and primary rat Schwann cells (RSC). However, merlin from rat newborn fibroblasts (RNF) and from a rat Schwann cell line (S-16) migrates at ~72 kD (Figure 2c suggesting different modifications or alternative splicing.

Cellular localization of endogenous merlin

The similarity between merlin and the ERMs suggests that it may also associate with both membrane and cytoskeletal structures. Initial immunocytochemical studies of exogenous merlin overexpressed in COS-7 cells transfected with *NF2* cDNA have supported the localization of the protein at or near the cell membrane (den Bakker *et al.*, 1995). However, there has been no report to date of detection and localization of endogenous merlin, expressed at physiological levels. To determine the cellular localization of endogenous merlin, we have screened various cell lines with all of our anti-merlin antibodies by indirect immunofluorescence. In cell lines that express merlin as detected by Western blots, none of the affinity eluted polyclonal antibodies is able to detect endogenous merlin in paraformaldehyde fixed and permeabilized cells. By contrast, the 1C4 monoclonal antibody detects endogenous merlin in human fetal and adult fibroblasts as well as in various primary meningioma cells. The localization is predominantly in the motile parts of the cells such as ruffling and leading edges (Figure 3). No staining of these structures was detected in MN11 meningioma cells

which do not express merlin (Figure 3). Similarly, no such signal was detected when the monoclonal antibody was preabsorbed with the corresponding fusion protein immunogen or when two different heterologous isotype control monoclonal antibodies were tested (data not shown). Some cytoplasmic and perinuclear staining is seen in the fibroblasts and meningioma cells, respectively, including MN11, a negative control cell line that does not express merlin. Similar perinuclear staining was detected with the heterologous isotype controls indicating that it is largely non-specific. The mAb 1C4, like many ERM antibodies, fails to detect the endogenous protein in cells such as glioma (H238), and HeLa, cells in which expression is detected by immunoblot. This is a common phenomenon observed with the ERM proteins (Henry *et al.*, 1995), perhaps due to more diffuse localization or to masking of epitopes in certain cell types. Similarly, no staining is observed with the rat Schwann cell line (S-16), rat newborn fibroblasts (RNF), primary rat Schwann cells and NIH3T3 cells.

Merlin and F-actin co-localize within leading lamellae

The ERM proteins co-localize with actin in surface microvilli and membrane ruffles in some cell types (Franck *et al.*, 1993; Henry *et al.*, 1995). A binding site for F-actin in the carboxy-terminal 34 amino acids of ezrin is highly conserved in moesin and radixin, but is not present in merlin (Turunen *et al.*, 1994). To determine whether merlin and F-actin also co-localize within the cell, we performed double staining in

primary meningioma cells (MN12) employing mAb 1C4 to detect merlin, and rhodamine phalloidin to detect F-actin simultaneously. These two proteins

localize to the motile sites of the cells mainly at the ruffling edges (Figure 4a-c). Merlin did not co-localize with F-actin at the stress fibers.

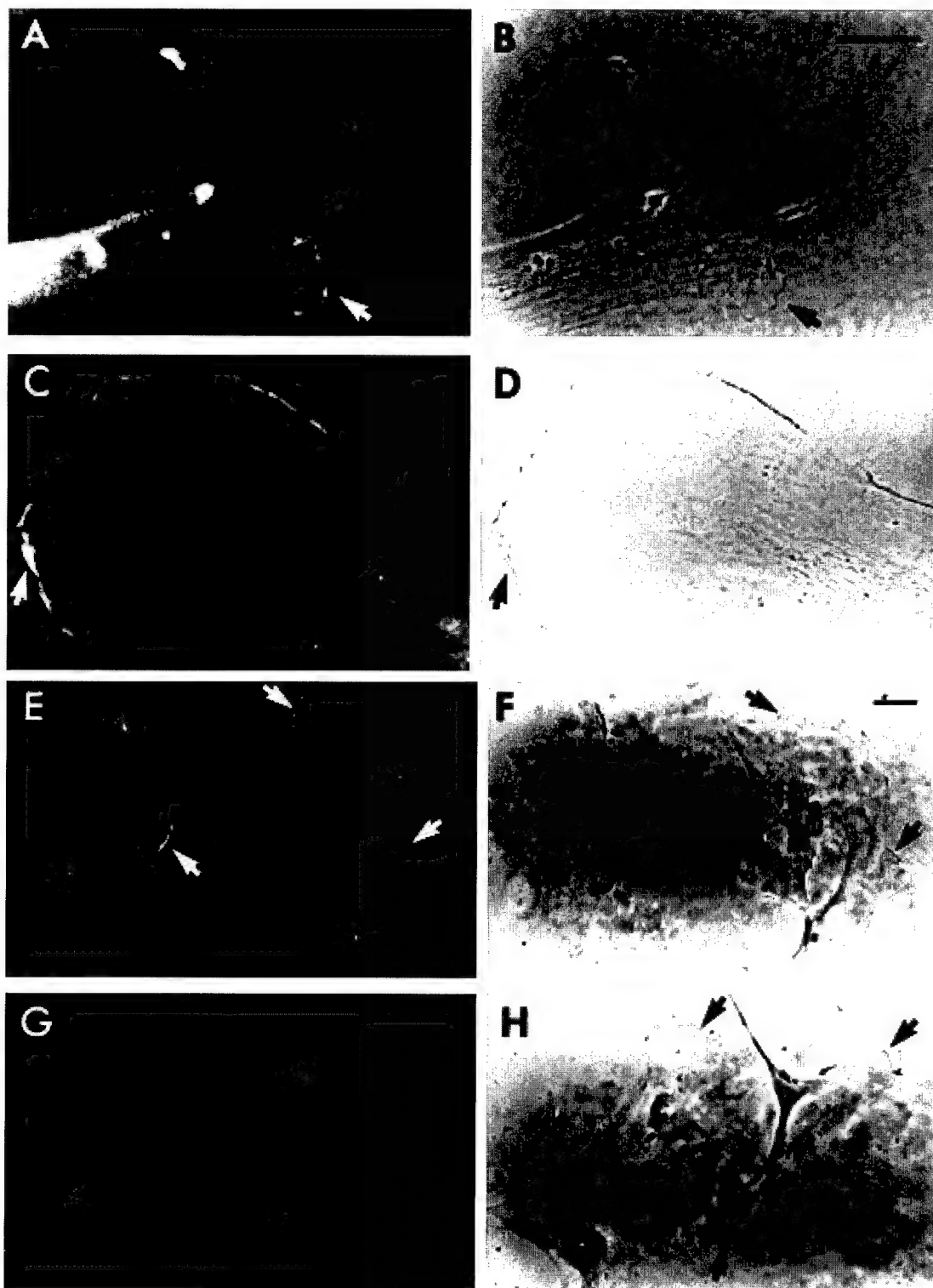


Figure 3 Subcellular localization of endogenous merlin. Cells were fixed with 4% paraformaldehyde, permeabilized and processed for indirect immunofluorescence experiments using the mAb 1C4. The endogenous localization of merlin is primarily detected in the motile parts of the cells such as ruffling membrane (arrows) in human fibroblasts (a, b) and primary meningioma cells (c, d). Panel e, f show multiple cells from MN12 with localization of merlin at membrane ruffles while panels g, h show the negative control cells MN11. Bar, 20 μ m



Figure 4 Co-localization of merlin with F-actin. a-c show the same primary meningeoma MN12 cell. Merlin was detected using the mAb 1C4 (b) and was simultaneously stained with rhodamine phalloidin for F-actin (c). Merlin and F-actin co-localize at the ruffling membrane (see arrows). Merlin does not co-localize with F-actin at the stress fibers. Bar, 20 μ m

Comparison of subcellular localization of merlin to ERM proteins

The ERM proteins localize to the ruffling membrane, microvilli, and filopodia among many other sites reported in various cell types. To determine whether merlin localizes to these structures, we compared their pattern of endogenous expression in human fibroblasts (RD136) and in meningeoma cells (MN12). In both these cell types, merlin localizes to the motile regions (Figure 3). The anti-ERM antibodies reveal only weak staining in the human fibroblasts, precluding direct comparison with merlin. However, in MN12 meningeoma cells, moesin localizes to the microvilli, and ezrin localizes to the filopodia, at the sites where two cells contact each other (Figure 5). Lack of co-localization of merlin and moesin in the same cell is shown in Figure 6. Merlin localizes specifically to the

ruffling edges, where no moesin staining is seen (Figure 6a-c). In a cell where moesin is detected clearly in the microvilli, merlin shows no staining of these structures (Figure 6d-f). As expected ezrin and merlin did not co-localize in individual cells (data not shown). Ezrin was seen mainly at sites of cell-cell contact, and such contacting cells do not display membrane ruffles. Consequently, they gave no specific merlin staining. Anti-radixin antibody consistently shows no significant staining. Thus, the localization of the ERM proteins moesin and ezrin in MN12 cells is quite distinct from that of endogenous merlin which localizes to the ruffling edges but never to filopodia or microvilli.

Discussion

Mutational analysis of the *NF2* gene (Gusella *et al.*, 1996) has clearly established that merlin is a tumor suppressor that results in tumor formation in a classic 'two-hit' inactivation model (Knudson, 1971). However, merlin's normal physiological role and its mechanism of action as a tumor suppressor have yet to be defined. As a close relative of ERMs, merlin represents a new class of tumor suppressor suggesting a role for membrane-cytoskeleton interactions in control of cell proliferation. Preliminary evidence has shown that merlin over-expression can inhibit cell growth (Lutchman and Rouleau, 1995), and can interfere with *Ras*-mediated transformation (Tikoo *et al.*, 1994) in NIH3T3 cells, but the biological significance of these phenomena in *NF2* is not at all clear. To support investigation of the biological function of this new class of tumor suppressor, we have developed a panel of polyclonal and monoclonal antibodies against merlin. Employing these antibodies, we have detected merlin at ~66 kD in many different cell types, a size similar to that predicted for the protein based on its cDNA sequence (Rouleau *et al.*, 1993; Trofatter *et al.*, 1993). This is in reasonable agreement with the report of Sainz *et al.* (1994) who estimated merlin at 65 kD in human sciatic nerve. However it differs significantly from the estimates of den Bakker *et al.* (1995) and Takeshima *et al.* (1994). The former authors reported that merlin immunoprecipitated as a 55 kD protein from various human tissues, but detected as a 70 kD protein when an *NF2* expression construct was transfected into COS-7 cells. Unfortunately these investigators did not report the expression of endogenous merlin in these cells. Takeshima *et al.* (1994) estimated merlin at 72 kD in various cultured human cell lines. However, these investigators raised their antisera against an immunogen containing the protein 4.1 domain raising the possibility that the antibody could cross react with other ERM family members.

Erzin, radixin, and moesin all migrate more slowly (apparent MW ~75 to 82 kD) on SDS gels than predicted by their respective cDNA sequences (predicted 67 to 69 kD), a feature mapped to the COOH-terminal half of the proteins. By contrast, in our hands merlin does not display anomalous electrophoretic behaviour, either in cell extracts or when expressed from constructs in either bacterial or mammalian cells. Thus, despite the predicted similarity in secondary structure, the difference in electrophoretic behaviour

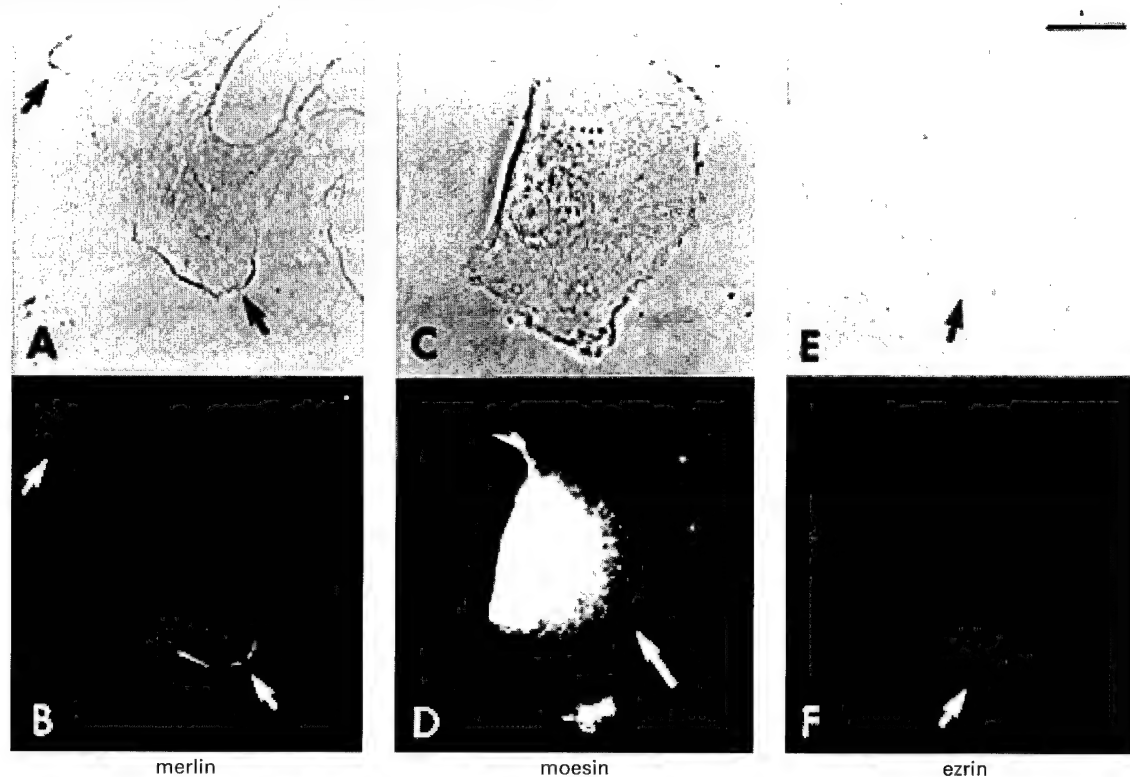


Figure 5 Localization of merlin compared to that of moesin and ezrin in meningioma cells. Indirect immunofluorescence experiments were performed to detect merlin using the mAb 1C4 (a, b); for moesin (c, d) and ezrin (e, f) affinity purified anti-moesin Ab#454 and affinity purified anti-ezrin Ab#464 were used. In MN12 meningioma cells, moesin localizes to the microvilli (c, d) and ezrin localizes to the filopodia (e, f), at the sites where two cells contact each other. Endogenous merlin localizes to the ruffling edges (a, b) but not to the microvilli or filopodia (see arrows). Bar, 30 μ m.

suggests a difference in the structural characteristics of merlin that may reflect a function distinct from the ERMs.

Merlin is expressed in a wide variety of cell types, based on our screening with a number of specific antibodies, in particular the mAb 1C4. Although the immunoblots are not strictly quantitative, the relative level of merlin expression is quite variable based on gels with equal quantities of protein loaded. Adult and fetal human fibroblasts, and certain primary meningioma cells exhibit a relatively high level of expression, whereas HeLa cells produce relatively less merlin. Interestingly, meningioma is a tumor type often associated with *NF2* gene mutations, both in *NF2* patients and in the general population. Since the vast majority of the inactivating mutations truncate the protein, most meningiomas are expected to possess little or no merlin. Indeed, primary meningioma cells (MN11) obtained from an *NF2* patient express no merlin detected by mAb 1C4. The germline mutation in this patient is a single-base change in codon 182 that introduces a stop codon; the somatic mutation in the other allele has not been identified (MacCollin and Gusella, unpublished data). The N-terminal polyclonal antibody N21 does not identify the predicted truncated protein of ~20 kD in this case (data not shown), suggesting that the truncated protein may be unstable. While *NF2* gene mutations can cause sporadic meningioma, there is considerable evidence that in a subset of this tumor type the *NF2* gene is not disrupted

and tumor formation is presumably due to lesions elsewhere in the genome (Ruttledge *et al.*, 1994; Wellenreuther *et al.*, 1995). In our examples of such meningiomas (e.g., MN12 and MN27), merlin is expressed at relatively high levels and is easily detected in immunofluorescence studies, suggesting that its function may be particularly important in meningeal cells.

Merlin is a very highly conserved protein, and consequently the mAb 1C4 also detects it in rodent cells such as, rat (S-16, primary rat Schwann cells, rat newborn fibroblasts) and mouse (NIH3T3) cell lines. In most cases the protein is of similar size to human merlin (~66 kD), except in rat newborn fibroblasts and the rat Schwann cell line S-16 where the protein migrates slightly slower. A similar difference in the electrophoretic mobility of rat merlin from rat fibroblast cells has been reported previously (Takeshima *et al.*, 1994) and may be due to post-translational modification, or novel alternative splicing of *NF2* in these cells. Similar size differences in ERM proteins among different species have also been observed (Berryman *et al.*, 1993; Sato *et al.*, 1992; Winckler *et al.*, 1994).

The primary localization of merlin to membrane ruffles in human primary meningioma cells and adult fibroblast cells is reminiscent of the reported ERM localization in some cell types (Birgbauer, 1991; Sato *et al.*, 1992) suggesting a functional similarity consistent with the predicted structural similarity between merlin

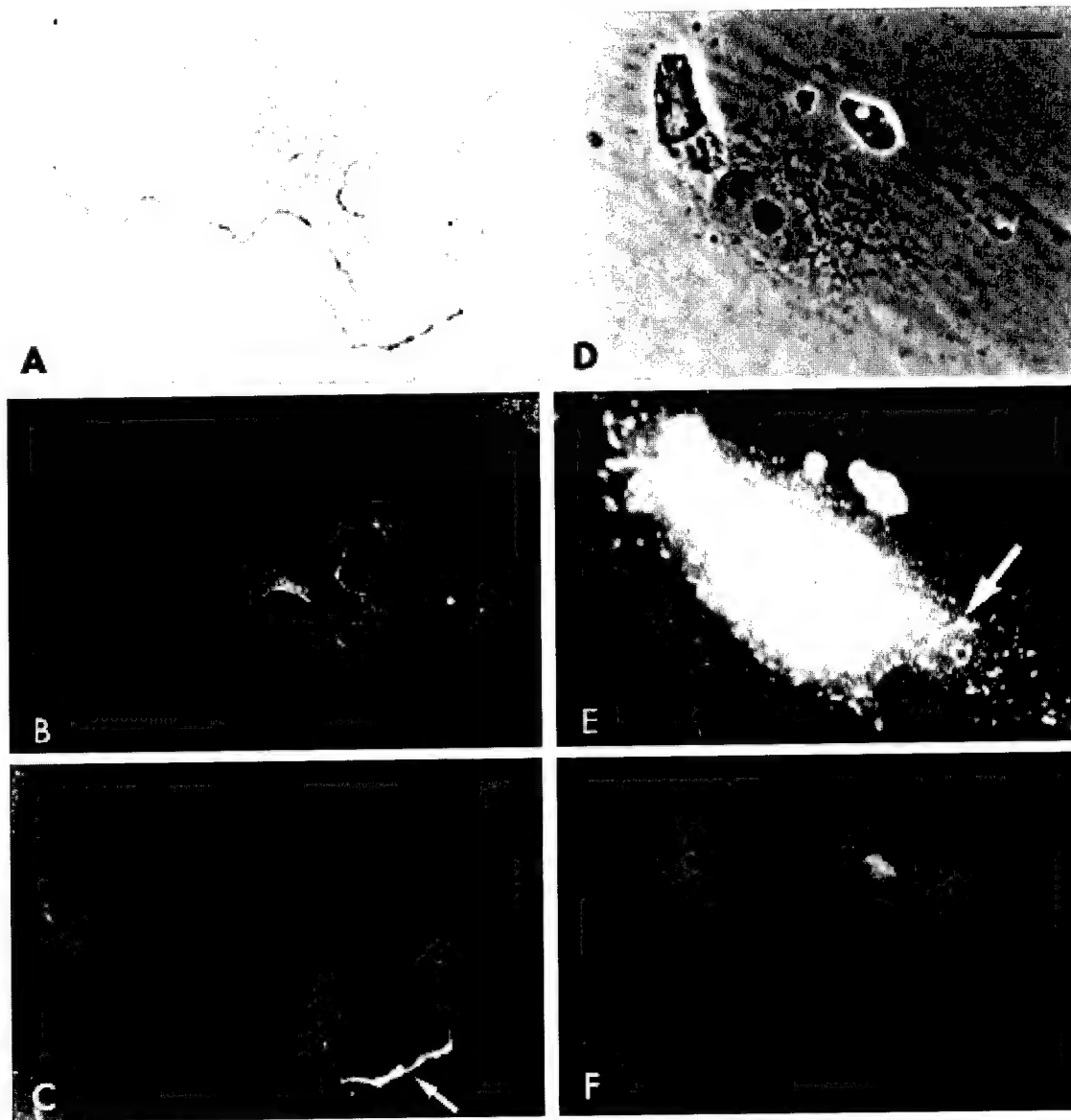


Figure 6 Lack of co-localization of merlin and moesin in MN12. Panels **a-c** show the same primary MN12 meningeoma cell. Merlin is detected at the ruffling edge (**c**, arrow) where moesin is not observed (**b**). Panels **d-f** show another MN12 cell where moesin is detected at the microvilli (**e**, arrow) where merlin is absent (**f**). Bar, 10 μ m

and ERMs. However, our direct demonstration that the localization of merlin is quite distinct from ezrin and moesin in primary meningeoma cells indicates that whereas the proteins may have related functions, the role of merlin in this cell type, where it is known to act as a tumor suppressor, is distinct. The co-localization of merlin with F-actin at the ruffling edges suggests an interaction between the two proteins. The carboxy terminal region of ezrin interacts directly with F-actin *in vitro*, and the region corresponding to the actin binding site is highly conserved in moesin and radixin, but not in merlin (Pestonjamasp *et al.*, 1995; Turunen *et al.*, 1994). It is possible that merlin encodes a distinct actin binding site at its carboxy-terminus, that actin binding is mediated by some other part of the protein, or that any interaction with actin is indirect, mediated by another protein.

Tumor formation due to inactivation of merlin probably results from perturbation of a signal transduction pathway that induces the target cell to grow and divide. The normal function of this pathway could involve such tasks as monitoring the state of the cytoskeleton or passing information from membrane signals simultaneously to the nucleus and the cytoskeleton. Merlin's localization suggests that it may be involved in the regulation or process of membrane ruffling, a common event observed in many cell types, often in response to certain extracellular factors. Membrane ruffles are particularly seen at the leading edges of motile cells where they are believed to be required for directed cell migration. Several previously characterized signal transducing molecules are implicated in the membrane ruffling response (Ridley, 1994). Studies have demonstrated the role for small GTP-

binding proteins *Rho* and *Rac* in the formation of the membrane ruffles and the reorganization of the cytoskeleton (Ridley *et al.*, 1992; Takaishi *et al.*, 1995). ERM family members have been reported to co-localize with *Rho* in a number of cellular locations, including TPA-induced membrane ruffles in MDCK2 cells (Takaishi *et al.*, 1995).

Whether *Rho* and *Rac* play a role in NF2 tumor formation remains to be determined, but it is likely that merlin is important for cell movement, shape or communication. Investigation of these processes in the Schwann cells and meningeal cells that are the targets of NF2 mutations may represent a fruitful avenue to elucidate the mechanism underlying the tumor suppressor function of merlin.

Materials and methods

Plasmid construction

For bacterial expression, three different segments of merlin were expressed as GST fusion proteins from pGEX2T (Pharmacia). GST-MERN21 spans residues 1–332, GST-MERC26 spans residues 308–579, and GST-MERC22 spans residues 308–590 (isoform 2) of merlin. GST-MERN21 and GST-MERC26 were made by PCR amplification of clone JJR1 (Trofatter *et al.*, 1993) and GST-MERC22 was amplified by RT-PCR from an adult human fibroblast RNA. The set of oligonucleotide primers employed were designed to have a BamHI site at the 5' end and an EcoRI site at the 3' end. PCR amplifications were performed with Pfu polymerase (Stratagene), the amplified fragments were digested with BamHI and EcoRI and subcloned into the pGEX2T vector. The entire sequences of the inserts were confirmed to be identical to the original published sequence (Trofatter *et al.*, 1993).

Expression and purification of GST-fusion proteins

Expression and purification of GST-fusion proteins were performed essentially as described (Smith and Johnson, 1988) with minor modifications. Briefly, logarithmically growing cultures of *E. coli* BL21 transformed with the pGEX2T recombinants were induced with 0.05 mM isopropyl-b-D-thiogalactopyranoside (IPTG) overnight at room temperature. The cells were pelleted, resuspended in PBS, incubated with 1.5 mg/ml of lysozyme in PBS for 15 min at 4°C followed by three freeze/thaw cycles. Debris was removed by centrifugation at 16 000 g for 30 min at 4°C. The soluble supernatant was then incubated with a 50% slurry of glutathione Sepharose 4B (Pharmacia) at room temperature for 1 h followed by incubation with bovine thrombin (Sigma) for 2 h to remove the GST affinity tail.

Generation of polyclonal and monoclonal antibodies

We have produced three anti-peptide antisera directed against the following regions of merlin: MP4, amino acid 354–367 (DELERRLLQMKEEA); MP5 amino acid 417–430 (IRTEEEKRLMEQKV); MP7, amino acid 581–595 (isoform 1) (TLQSAKSRVAFEEEL). An extra N-terminal Cys was added to these peptides for coupling. The peptide synthesis, immunization of rabbits, and bleeding were performed by Multiple Peptide System (San Diego, CA). We also produced antibodies for the bacterially expressed GST-MERN21 and GST-MERC26 in both rabbits and chickens. The immunization of rabbits and chickens, the

bleeds and the collection of eggs were performed by East Acre Biological (Southbridge, MA). The peptide and the fusion antibodies were affinity eluted from the bacterially expressed GST-MERN21 and GST-MERC26 transferred onto nitrocellulose employing the method of Olmsted (Olmsted, 1981) with slight modifications. For monoclonal antibody production, four mice were injected with 50 µg of thrombin cleaved carboxy-terminus merlin (GST-MERC22). After the final boost the best responders were sacrificed, spleens were removed and fused to SP2 myeloma cells. Fusions and the selection were carried out as described (Harlow and Lane, 1988). Class and subclass were determined using an isotyping kit (Amersham). The mAb 1C4 was found to be an IgG1, κ. For preabsorption experiments, 1C4 was incubated overnight with 75 µg of GST-MERC22 Sepharose beads and 75 µg of GST alone as a control.

Other antibodies

The polyclonal antibodies anti-ezrin (464), anti-radixin (457) and anti-moesin (454) raised against unique peptides corresponding to the mouse sequences (Winckler *et al.*, 1994) were generously provided by Dr Frank Solomon (MIT). The heterologous isotype control mAbs include the anti-MHC class II mAb 3B12 (Spertini *et al.*, 1992) and MOPC21 (Litton Bionetics, Charleston SC).

Cell lines and culture conditions

MRC-5 (fetal human fibroblast), RD136 (adult human fibroblast), H238 (glioma), HeLa, rat newborn fibroblast (RNF), and NIH3T3 cells were maintained in DMEM supplemented with 10% fetal calf serum (FCS). The rat Schwann cell line S-16 was generously provided by Dr Quarles and maintained as described (Goda *et al.*, 1991). Human primary meningioma cells MN11, MN12, MN27 were cultured as described (Logan *et al.*, 1990) and analysed after less than five passages. They were analysed as subconfluent cultures with a doubling time of ~4 days. MN12 derived from a menigothelial meningioma and MN27 from a transitional meningioma. Histology was not available from MN11. Primary rat Schwann cell cultures were initiated from newborn rat sciatic nerve as described (Brookes *et al.*, 1979).

Western blot analysis

Total cell extracts were prepared from different cell types described above. After washing the cells twice with PBS, the cell pellet was resuspended in PBS with protease inhibitors (0.04 U/ml aprotinin, 1 µg/ml leupeptin 2 mM pepabloc). Cells were lysed by adding one half volume of 3 × GSD (33% Glycerol, 6% SDS, 300 mM DTT) sample buffer. Protein concentration was determined using the DC protein assay system (Bio-Rad, Melville NY). Lysates were fractionated on a 7.5% or 10% polyacrylamide gel according to the method of (Laemmli, 1970), electrophoretically transferred to nitrocellulose (Bio-Rad) and stained for total protein with 0.2% Ponceau-S (Sigma Chemical Co., St. Louis, MO). The blots were probed with either anti-merlin or ERM antibodies and the proteins were visualized with horseradish peroxidase conjugated secondary antibodies and ECL system (Amersham). Affinity purified polyclonal antibodies were used at an equivalent dilution of 1:200. The monoclonal antibody was used at a dilution of 1:2500 (1 ng/µl).

Immunofluorescence

Cells were fixed with 4% paraformaldehyde in PBS for 30 min at 37°C, washed three times with PBS, permeabi-

lized with 0.1% NP40/PBS for 15 min at room temperature and blocked with 10% normal goat serum in PBS for 45 min at 37°C. The first and second antibodies were diluted in 0.1% BSA/PBS and incubated at 37°C for 1 h and 30 min, respectively. As primary antibodies we used the monoclonal 1C4 against merlin (1:100 dilution), and the affinity purified anti-ERM polyclonal antibodies as described (Henry *et al.*, 1995; Winckler *et al.*, 1994). As secondary antibody we used goat anti-mouse FITC conjugated (TAGO). To visualize F-actin, we used rhodamine-coupled phalloidin (1:2000) (Molecular Probes, Eugene OR).

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Frequency and Distribution of NF2 Mutations in Schwannomas

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Sporadic and inherited schwannomas were scanned for the nature, frequency, and distribution of mutations in the *NF2* locus encoding the merlin tumor suppressor protein on 22q. Of 58 tumors, 47% displayed loss of heterozygosity for *NF2*, leaving a total of 89 *NF2* alleles to be examined. Pathogenic alterations were identified in 62 of these alleles, including 36 frameshifts with premature termination, 14 nonsense mutations, and 12 changes presumed to affect splicing. Effects of ten of the latter were confirmed in the *NF2* transcript and indicated that activation of cryptic splice sites in coding sequence is another frequent mechanism leading to truncation of merlin. The mutations were relatively evenly distributed across both the protein 4.1 superfamily (exons 1-9) and the α -helical (exons 10-15) domains of merlin, but they did not occur at all in exons 16 and 17, which encode the protein's alternative COOH-termini. The data support the "two-hit" tumor suppressor model for formation of schwannomas and indicate that loss of merlin function can be achieved by truncation at various locations in the protein. However, the absence of mutations in exons 16 and 17 suggests that an inactivating mutation affecting only one of the merlin's alternative termini may not be sufficient to eliminate tumor suppressor function. *Genes Chromosom Cancer* 17:45-55 (1996).

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INTRODUCTION

Neurofibromatosis type 2 (NF2) is a severe autosomal dominant genetic disorder that is characterized by abnormalities of the cells derived from the neural crest (Martuza and Eldridge, 1988; Short et al., 1994). It is associated with the development of several types of nervous system tumors, including bilateral vestibular schwannomas, spinal nerve root schwannomas, meningiomas, and, less frequently, ependymomas. The molecular cloning of the *NF2* gene in 1993 (Rouleau et al., 1993; Trofatter et al., 1993) revealed its product, called merlin (schwannomin), to be similar to members of the ezrin-radixin-moesin (ERM) family of proteins that have been postulated to link the cytoskeleton and the cell membrane (Gould et al., 1989; Funayama et al., 1991; Lankes and Furthmayr, 1991). Like the ERM proteins, merlin possesses a protein 4.1 superfamily domain followed by a long stretch of α -helical structure and a charged COOH-terminus, although alternative splicing produces alternate versions of the latter.

A number of germline mutations in the *NF2* gene have been reported, confirming that inactivation of merlin underlies NF2 (Bourn et al., 1994; MacCollin et al., 1994; Mérel et al., 1995a; Sainz et al., 1995). The "two-hit" model for this tumor suppressor has also received support from analysis of sporadic tumors of the types most often associated with NF2, including schwannomas (Bijlsma et al., 1994; Irving et al., 1994; Jacoby et al., 1994;

Lekanne Deprez et al., 1994; Sainz et al., 1994, 1995; Twist et al., 1994; Mérel et al., 1995b) and meningiomas (Ruttledge et al., 1994; Mérel et al., 1995b; Wellenreuther et al., 1995), but *NF2* mutations have been observed only rarely in ependymomas (Rubio et al., 1994; Mérel et al., 1995b). *NF2* mutations also occur with high frequency in one tumor that is unrelated to NF2, malignant mesothelioma (Bianchi et al., 1995; Sekido et al., 1995), and they may occur rarely in other non-NF2-related tumors, including melanoma, breast cancer, and colorectal cancer (Arakawa et al., 1994; Bianchi et al., 1994; Rustgi et al., 1995).

To delineate more precisely the spectrum of germline and somatic mutations occurring in schwannomas, we have used single-strand conformation polymorphism (SSCP) analysis and scanned all 17 exons of the *NF2* gene in 58 NF2-associated and sporadic schwannomas. We describe 62 previously unreported mutations plus two alterations that are most likely to be benign polymorphisms in *NF2*. Fifty of the mutations predict direct truncation of merlin due to introduction of either a nonsense codon (14) or a frameshift (36). Twelve mutations alter sequences around splice sites. Ten of these were confirmed by reverse transcription-

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polymerase chain reaction (RT-PCR) of mRNA from the corresponding tumors to affect splicing of *NF2*, sometimes in unexpected ways.

The various mutations of *NF2* that were detected in these tumors occurred in all exons except exons 16 and 17, encoding the alternative COOH-termini of merlin. These data support the model, holding that merlin inactivation is crucial to formation of schwannomas and that merlin can be inactivated by a wide variety of changes that do not cluster in a single part of the *NF2* gene. The absence of mutations affecting exclusively exon 16 or exon 17 also argues in favor of the hypothesis that inactivation of only one of the alternative C-termini may not be sufficient to eliminate merlin's tumor suppressor function.

MATERIALS AND METHODS

Tissue Samples

Tumor specimens were obtained at the time of surgery and were frozen for DNA or RNA analysis. Blood samples were also obtained at the time of surgery to serve as normal tissue controls. High-molecular-weight DNA was extracted from peripheral blood leukocytes and from frozen pulverized tumor tissue by sodium dodecyl sulfate (SDS)-proteinase K digestion followed by phenol and chloroform extractions (Seizinger et al., 1986). Total cellular RNA was extracted by a single-step method using RNA STAT-60 (Tel-Test, Friendswood, TX). Pathology reports were reviewed for all tumors to document patient sex, age, and tumor location. Patients were given a diagnosis of NF2 based on NIH criteria (Mulvihill et al., 1990), as modified by Evans et al. (1992). Three of the seven NF2 patients had somewhat atypical disease, in that only unilateral vestibular schwannomas were present after the age of 30 years. A single patient carried a diagnosis of schwannomatosis by the criteria of MacCollin et al. (1996). Tumors S114 and S120 were from patients 5095 and 2815, which were reported in MacCollin et al. (1994), and tumor S125 was from patient 4 in MacCollin et al. (1996).

SSCP Analysis and DNA Sequencing

SSCP analysis was performed as described (Jacoby et al., 1994), with the use of published PCR conditions and the primer pairs listed in Table 1. All 17 exons of the *NF2* gene, including 250 bp beyond the stop codon, were screened. The amplified products were separated on 6 or 8% nondeaturing polyacrylamide gels containing 8% glyc-

erol overnight at 6–8 W or on 0.5X MDE (AT Biochem) gels overnight at 6 W. Products showing mobility shifts were sequenced directly by the dideoxy-chain termination method with Sequenase (T7 DNA polymerase, Amersham) used as described previously (Jacoby et al., 1994). Alternatively, amplified products were cloned into a T-vector (pCRII, Invitrogen, or pT7Blue; Novagen) and sequenced as above.

Loss of Heterozygosity Analysis

Genomic DNA was amplified by using primer pairs for two polymorphic microsatellite markers proximal to NF2, D22S193 (Genome Data Base, version 5.6) and D22S275 (Weissenbach et al., 1992); one intragenic marker, D22S929 (Bourn and Strachan, 1995); and two markers distal to NF2, D22S268 (Marineau et al., 1993) and D22S430 (Sainz et al., 1993). PCR products were analyzed as reported previously (Jacoby et al., 1994).

RT-PCR

First-strand cDNA was synthesized from 1–2 µg of total cellular RNA with oligo(dT)_{12–18} (Gibco-BRL) used as primer and murine Moloney leukemia virus reverse transcriptase, as recommended by the supplier (Gibco-BRL). Full-length *NF2* cDNA was amplified with 1 µl of the reverse-transcribed product in a final volume containing 50 µl of 200 µM each of dATP, dCTP, dGTP, and dTTP; 20 pmol each of primers A1 and D2; 2.5 units of Taq polymerase (Perkin-Elmer); and 10 mM Tris, pH 8.3, 1.5 mM MgCl₂, 50 mM KCl, and 0.01% gelatin. PCR reactions were carried out at 94°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute for 30 cycles, followed by 72°C for 10 minutes. For each tumor, RT-PCR was also carried out with the use of random hexamers [pd(N)₆; Pharmacia] to prime cDNA synthesis, followed by amplification of full-length cDNA with primers A1 and D3. Nested PCR was then performed as above with 1 µl of full-length product used in a final volume of 50 µl with the following primer pairs: A1-A2, B1-B2, C1-C2, or D1-D2. Primers used for RT-PCR analysis included A1 (bp 16) 5'GCCTGAGCCCCGC-GCCATGGCCGGG3' (sense; Pykett et al., 1994), A2 (bp 506) 5'AGCAATTCTCTTGGGCCAA3' (antisense), B1 (bp 404) 5'CTGAGGCTTCTGTCTCCTG3' (sense), B2 (bp 878) 5'TTAACACGAAGCTTTGAGGAG3' (antisense), C1 (bp 784) 5'CGAAACATCTCGTACAGTGAC3' (sense), C2 (bp 1375) 5'GCAGGTCTGCTTCAGCTG3' (antisense), D1 (bp 1314) 5'GAAGATGGCTGAGGAG-

TABLE 1. Primers for Neurofibromatosis Type 2 (NF2) Exon Amplification

Exon ^a	Product (bp)	Temp (°C) ^b	Primer 1 (5'–3')	Primer 2 (5'–3')
1	236	58	GCTAAAGGGCTCAGAGTGCAG	GAGAACCTCTCGAGCTTCCAC
2	243	60	AGTGCAGAGAAAAGGTTTTATTAATGAT	TGGAAAAGCTCACGTCAGCC
3	275	60	GCTTCTTTGAGGGTAGCACA	GGTCAACTCTGAGGCCAACT
4	188	60	CCTCACTTCCCCTCACAGAG	CCCATGACCCAAATTAACGC
5	172	58	TGGCAGTTATCTTTAGAATCTC	TTAGACCACATATCTGCTATG
6	181	58	AAACAATACCAAATTTACTTCATG	GCCCATAAAGGAATGTAAACC
7	174	58	CCATCTCACTTAGCTCCAATG	CTCACTCAGTCTCTGTCTAC
8	248	60	GAAGGTTGAATAAAATTTTGAGCCTC	GACAGGGAAGATCTGCTGGACC
9	209	60	GGTTGCGCATTTGTGGAATT	CCATTATCAGTAATGAAAACCAGG
10	260	58	TGCTACCTGCAAGAGCTCAA	CTGACCACACAGTGACATC
11	219	60	GGTCTCGAGCCCTGTGATTCA	AGGAAGTCCCAAGTAGCCTCC
12A	161	60	CGGGAGAACAGCACATGATC	CGCTGCATTTCTGCTCAG
12B	284	58	GCTGAAAAGGCCAGATCA	CTTGAGGACAACTGCTGTAG
13	227	58	GGTGCTTTTCCCTGCTACCT	GGGAGGAAAGAGAACATCAC
14	197	60	CTGTGCTTGTATGACCCAAG	AGGGCACAGGGGGCTACA
15	244	60	TCTGCCCAAGCCCTGATGC	TGGTCCTGATCAGCAAAATAC
16	148	60	GGCATTGTTGATATCACAGGG	GGCAGCACCATCACCACATA
17	212	58	AGGACCCTGTAGACAGAG	CCAGCCAGCTCCTATGGATG ^c

^aAll exons were scanned by single polymerase chain reaction (PCR) assays except exon 12, where overlapping assays were required. Primers and PCR conditions for seven of these exons (1, 3, 4, 10, 12B, 13, and 16) were described in Jacoby et al. (1994).

^bAnnealing temperature for PCR reaction.

^cThis primer is missing one base relative to the genomic sequence.

TCAG3' (sense), D2 (bp 1886) 5'CCAGCCAGCT-CCTATGGATG3' (antisense), and D3 (bp 2334) 5'ACTTGCTGGTACAGAGCTCT3' (antisense). Amplified products were analyzed on 1.8% agarose gels. Aberrant products were excised from gels and sequenced directly as described above. Alternatively, where resolution of PCR products was poor on agarose gels, the products were cloned into a T-vector, and multiple colonies were sequenced on both strands.

Data Analysis

Correlations between the clinical history of the patient and the genotype of the schwannoma were examined with the aid of the Data Desk Analysis package (Data Description, Inc., Ithaca, NY).

RESULTS

Loss of Heterozygosity on Chromosome 22

We examined 58 schwannomas, including 50 sporadic tumors and eight tumors obtained from patients with NF2 or related syndromes. Most (53) of the tumors were vestibular schwannomas. Five tumors (S101, S68, S125, S74, and S98) originated from locations in the nervous system other than the vestibular nerve. Blood and tumor DNAs were genotyped with four polymorphic DNA markers flanking the *NF2* gene and one intragenic marker.

All five markers were tested on each tumor, and at least one marker was informative for every tumor (data not shown). The loss of heterozygosity (LOH) analysis indicated that 27 tumors (47%) had lost one *NF2* allele. Thus, a total of 89 independent *NF2* alleles remained to be examined for *NF2* mutations.

SSCP Analysis

The 58 schwannomas were examined by using SSCP analysis for mutations in *NF2*. The coding sequences of all 17 exons were scanned as well as 250 bp of the 3' untranslated region. DNA from the corresponding blood samples was included for comparison in each case. Both normal and altered products were compared by direct DNA sequence analysis to identify the precise base changes. Shifts in mobility were observed in 63 instances. Sixty mobility shifts were detected after separation of single strands of DNA on polyacrylamide gels, and three additional shifts were detected after the same DNAs were fractionated on the MDE gel matrix. Among the SSCP shifts, 57 of the 63 were somatic in origin, whereas six were also present in the paired blood sample.

NF2 Mutations in Schwannomas

Sequence analysis of exons producing abnormal SSCP patterns revealed a total of 64 alterations in

TABLE 2. NF2 Mutations in Patients with NF2 or Related Syndrome

Number	Patient diagnosis ^a	Sex and age ^b	Tumor location ^c	Exon	Nucleotide change ^d	Codon change ^e	Consequence	Origin ^f	Alleles ^g
S68	NF2	F 40	Spinal	—	—	—	—	—	2
S99	NF2	F 35	VS	E7	675 + 5 G→A	—	Splice donor	G	2
				E10	932* del 1 bp (G)	Arg311→321X	Frameshift	S	
S101	NF2	M 39	Femoral nerve	E15	1737 + 12 ins 220 bp	—	Splice donor	G	1
S102	NF2	M 50	VS	—	—	—	—	—	2
S114	NF2	M 20	VS	E2	169 C→T	Arg57X	Nonsense	S	2
				E11	1,021 C→T	Arg341X	Nonsense	G	
S116	NF2	M 26	VS	—	—	—	—	—	1
S120	NF2	M 25	VS	E8	784 C→T	Arg262X	Nonsense	G	2
				E11	1,040 del 1 bp (A)	Glu347→363X	Frameshift	—	
S125	Schwannomatosis	M 17	Fifth cranial nerve	E2	233 ins 33 bp and del 1 bp (A)	Asp78→122X	Frameshift or splice donor	S	2
				E8	784 C→T	Arg262X	Nonsense	S	

^aDiagnosis of neurofibromatosis type 2 (NF2) by NIH criteria (Mulvihill et al., 1990), as modified by Evans et al. (1992). In patients S68, S99, and S102, only unilateral vestibular schwannomas were present at ages above 30 years.

^bM, male; F, female.

^cVS, vestibular schwannoma.

^dAn asterisk indicates that the precise start position of a deletion (del) or insertion (ins) is uncertain. In these cases, the first possible nucleotide position is given. Intronic mutations are designated as + or - relative to the first or last nucleotide of the nearest exon. The E11 mutation in S114 and the E8 mutation in S120 were reported by MacCollin et al. (1994).

^eX, nonsense mutation.

^fS, somatic mutation; G, germline mutation.

^gNumber of NF2 alleles in tumor predicted by heterozygosity testing with chromosome 22 microsatellite markers.

the NF2 gene sequence; in one tumor, the shift led to delineation of two mutations in the same exon. Sixty-two of the 64 alterations were predicted to alter the protein product, whereas 2 were predicted to have no effect (Tables 2 and 3). Germline mutations were detected in 4 of 7 patients with a confirmed diagnosis of NF2 (Table 2), whereas none was found among 51 patients without NF2 (Tables 2 and 3). The 4 germline mutations included two nonsense mutations in exons 8 and 11 (S114 and S120) and 2 intronic mutations; one was a large 220 bp insertion at position 1737+12 in intron 15 (S101), and the other was at position 675+5 in intron 7 (S99).

Single somatic mutations were observed in schwannomas from 3 of 7 patients with NF2 (S99, S114, and S120), whereas the tumor examined from the one patient with schwannomatosis (S125) contained two somatic mutations (Table 2). For the sporadic tumors, a total of 53 somatic mutations were identified in 43 tumors (Table 3). Consistent with our previous study (Jacoby et al., 1994), the most frequent somatic lesions observed (33 of 58; 57%) were small deletions of 1–52 bp that

produced frameshifts, causing truncated protein with altered sequence, or that were presumed to affect splicing. Seven insertions of 1–8 bp that caused frameshifts were also observed. The remaining 18 somatic changes were point mutations that produced stop codons (11) or that occurred near or within the splice donor (3) or acceptor (4) sites.

No missense mutations were observed; however, two other DNA alterations were detected that may represent rare polymorphisms. In patient S79, a single base change from G to A at position 1,792, four bases beyond the termination codon, occurred in both normal and tumor tissue. In patient S98, both blood and tumor tissue contained a silent C to T transition in alanine codon 98. Because neither of these changes is expected to have an effect on the NF2 transcript or protein, and because they are germline differences in patients without a diagnosis of NF2, they may be rare benign polymorphisms rather than mutations in the NF2 gene. Neither patient was known to harbor more than one tumor, and no family members were available for comparison.

TABLE 3. NF2 Mutations in Sporadic Schwannomas^a

Number	Sex and age	Tumor location	Exon	Nucleotide change	Codon change	Consequence	Origin	Alleles
S65	F 43	VS	E9	852* ins 1 bp (T)	Lys284→292X	Frameshift	S	1
S66	F 53	VS	E13	1,396 C→T	Arg466X	Nonsense	S	1
S67	F 44	VS	E2	169 C→T	Arg57X	Nonsense	S	2
			E2	115-15 G→A	—	Splice acceptor	S	
S69	F 34	VS	E4	424-431 del 8 bp	Ala142→151X	Frameshift	S	2
			E13	1,408 del 1 bp (C)	Gln470→484X	Frameshift	S	
S70	M 63	VS	—	—	—	—		1
S71	F 19	VS	E3	339 del 1 bp (C)	Ile113→122X	Frameshift	S	1
S72	M 30	VS	E4	380-388 del 9 bp and 378 T→C	Leu127X	Nonsense	S	2
			E11	1,021 C→T	Arg341X	Nonsense	S	
S73	F 69	VS	E5	516+1 G→A	—	Splice donor	S	1
S74	M 42	Spinal	E10	955 del 1 bp (C)	Gln319→321X	Frameshift	S	1
S75	F 43	VS	E4	399* del 1 bp (C)	Cys133→173X	Frameshift	S	2
			E14	1,571-1,574 del 4 bp	Glu524→549X	Frameshift	S	
S76	M 40	VS	E7	635-657 del 23 bp and 634 C→A	Gln212→226X	Frameshift	S	2
			E10	981-999+17 del 36 bp	—	Splice donor	S	
S77	M 77	VS	E7	642-667 del 26 bp	Leu214→226X	Frameshift	S	2
S78	F 20	VS	E3	316 G→T	Glu106X	Nonsense	S	1
S79	F 39	VS	E1	37-88 del 52 bp	Ser13→39X	Frameshift	S	2
			E17	1,792 G→A	—	Polymorphism?	G	
S80	M 59	VS	—	—	—	—		1
S81	M 60	VS	—	—	—	—		1
S82	F 50	VS	E5	472-473* del 2 bp (CA)	His158→201X	Frameshift	S	2
			E13	1,362-1,390 del 29 bp	Leu454→493X	Frameshift	S	
S83	M 56	VS	—	—	—	—		2
S84	M 37	VS	E12	1,327-1,340+2* del 16 bp	—	Splice donor	S	2
S85	M 59	VS	E11	1,084 C→T	Gln362X	Nonsense	S	2
S86	F 71	VS	E12	1,189-1,195* del 7 bp	Leu397→425X	Frameshift	S	1
S87	F 33	VS	E12	1,124 del 1 bp (T)	Met375→425X	Frameshift	S	1
S88	F 34	VS	E3	304* del 1 bp (C)	Pro102→122X	Frameshift	S	1
S89	F 44	VS	E13	1,375-1,409* del 35 bp, ins 7 bp	Gln459→484X	Frameshift	S	1
S90	F 62	VS	E7	643-656* del 14 bp	Glu215→226X	Frameshift	S	2
S91	F 69	VS	—	—	—	—		2
S92	M 30	VS	E7	673-675+11* del 14 bp	—	Splice donor	S	1
S93	M 31	VS	E3	276-282* del 7 bp	Val92→122X	Frameshift	S	2
S94	F 70	VS	E12	1,340+1 G→A	—	Splice donor	S	1
S95	F 46	VS	E3	342* ins 8 bp	Thr114→122X	Frameshift	S	2
			E10	942* del 1 bp (T)	Asp314→321X	Frameshift	S	
S96	M 45	VS	E1	70 ins 1 bp (T)	Val24→47X	Frameshift	S	1
S97	M 44	VS	E13	1,347-1,387* del 41 bp	Lys449→493X	Frameshift	S	1
S98	M 28	Jugular foramen	E3	294 C→T	Ala98Ala	Polymorphism?	G	2
S100	M 40	VS	E8	781 ins 7 bp	Ile261→267X	Frameshift	S	1
S103	M 51	VS	E13	1,357 del 1 bp (C)	Gln453→454X	Frameshift	S	2
S104	F 34	VS	E1	45 del 1 bp (G)	Lys15→24X	Frameshift	S	2
			E12	1,163* del 1 bp (C)	Ala388→425X	Frameshift	S	
S105	M 61	VS	E4	393* ins 4 bp	Ile131→135X	Frameshift	S	1
S106	F 54	VS	E5	448-1 G→A	—	Splice acceptor	S	1
S107	M 58	VS	E3	264* del 1 bp (G)	Lys88→122X	Frameshift	S	1

(continued)

TABLE 3. *NF2* Mutations in Sporadic Schwannomas^a (Continued)

Number	Sex and age	Tumor location	Exon	Nucleotide change	Codon change	Consequence	Origin	Alleles
S108	M 55	VS	E5	459 del 1 bp (C)	Tyr153X	Frameshift	S	2
			E8	784 C→T	Arg262X	Nonsense	S	
S109	M 57	VS	E11	1,022–1,031 del 10 bp	Arg341→345X	Frameshift	S	2
			E13	1,345 A→T	Lys449X	Nonsense	S	
S110	M 50	VS	E12	1,179–1,185* del 7 bp and 1,178* A→C	Glu393→425X	Frameshift	S	1
S112	F 67	VS	E6	528* ins 1 bp (T)	Leu176→201X	Frameshift	S	2
S113	F 65	VS	E13	1,396 C→T	Arg466X	Nonsense	S	1
S115	F 46	VS	E4	432 C→A	Tyr144X	Nonsense	S	1
S117	M 69	VS	—	—	—	—	—	2
S118	F 39	VS	E3	241–9 A→G	—	Splice acceptor	S	2
			E14	1,574+1 G→A	—	Splice donor	S	
S119	F 50	VS	E7	600–1 G→A	—	Splice acceptor	S	2
S121	M 44	VS	E14	1,570 del 1 bp (G)	Glu524→549X	Frameshift	S	1
S130	F 39	VS	—	—	—	—	—	2

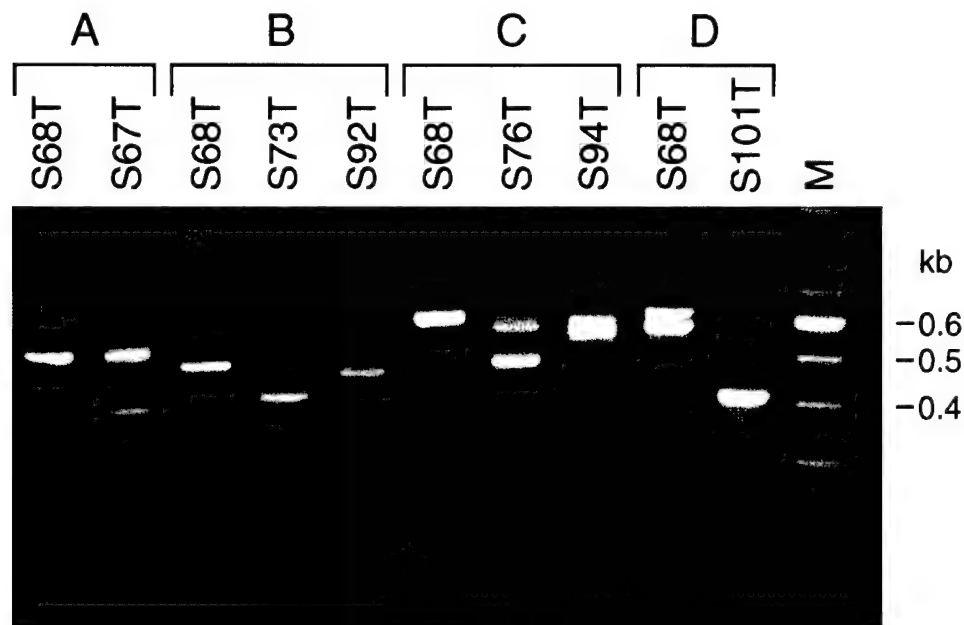
^aFor definitions of symbols, see Table 2 footnote.

Figure 1. Ethidium bromide-stained agarose gel of splice products obtained in reverse transcription-polymerase chain reaction (RT-PCR) analysis of six schwannomas containing splice defects in the neurofibromatosis type 2 (*NF2*) gene. Full-length cDNA was synthesized from total

cytoplasmic RNA followed by nested PCR of overlapping quadrants of the cDNA, including exons 1–5 (A), exons 4–9 (B), exons 8–13 (C), and exons 12–17 (D). RNA from patient S68T was included as a control in each case. M, marker.

TABLE 4. Splice Alterations in NF2 cDNAs From Schwannomas^a

Genomic NF2 mutation			Major altered cDNA products	
Tumor	Exon	Predicted consequence	Nucleotide change	Consequence
S67	E2	Splice acceptor	115-240 del 126 bp (E2)	Del 42 aa (Met39 to Lys80)
S106	E5	Splice acceptor	448-467 del 20 bp	Frameshift Tyr150→201X
S73	E5	Splice donor	448-516 del 69 bp (E5)	Del 23 aa (Tyr150 to Arg172)
S119	E7	Splice acceptor	600-675 del 76 bp (E7)	Frameshift Arg200→250X
S92	E7	Splice donor	648-675 del 28 bp	Frameshift Met216→250X
			653-675 del 23 bp	Frameshift Gly218→226X
S99	E7	Splice donor	653-675 del 23 bp	Frameshift Gly218→226X
S76	E10	Splice donor	886-999 del 114 bp (E10)	Del 38 aa (Ile296 to Gln333)
S84	E12	Splice donor	1,288-1,340 del 53 bp	Frameshift Val430→493X
			1,303-1,340 del 38 bp	Frameshift Val435→493X
S94	E12	Splice donor	1,288-1,340 del 53 bp	Frameshift Val430→493X
			1,303-1,340 del 38 bp	Frameshift Val435→493X
S101	E15	Splice donor	1,575-1,782 del 208 bp (E15,E16)	Frameshift Lys525→613X

^aReverse transcription-polymerase chain reaction (RT-PCR) was performed on total cellular RNA as described in Materials and Methods. For definitions of symbols, see Table 2 footnote.

Effect of Splice Mutations on NF2 Transcript

Twelve *NF2* mutations were predicted to affect splicing of the *NF2* transcript either because they directly disrupted the consensus splice signals or because they were located in their vicinity. Tumor RNA was available for testing the effects of ten of these mutations on the *NF2* transcript by using RT-PCR. Major alternatively spliced transcripts that were consistent with the genotype of the corresponding tumors were observed in all cases (Fig. 1, Table 4). Additional minor splicing products may have been present in some tumors, but only major variants were isolated and sequenced in this study. The splice alterations caused in-frame deletions in three tumors and frameshifts with premature termination in the seven others, as indicated in Table 4.

Disruption of a splice donor site resulted in the complete absence from the transcript of the upstream exon sequences in two cases (S73, S76) and resulted in the use of one or more cryptic splice sites within the exon in four cases (S84, S92, S94, S99). Disruption of a splice acceptor site resulted in the absence of the subsequent exon in two tumors (S67, S119) and resulted in the use of a cryptic splice site in the exon in another tumor (S106).

The remaining intronic mutation also effected aberrant splicing of the *NF2* transcript, but it yielded a more complicated pattern. In tumor S101, a 220 bp insertion at position 1,737+12 resulted in the absence of the upstream exon 15 sequences from the transcript. Interestingly, exon 16

sequences were also absent from the S101 cDNA, in contrast to the finding in most schwannomas, which yield approximately equal amounts of transcript with and without exon 16 under our conditions (Jacoby, unpublished observation). Thus, this alteration causes the absence of both upstream and downstream exons from the transcript.

Correlation Between Clinical Data and Genotype of Tumor

An attempt was made to correlate the results of the molecular genetic analysis with each patient's clinical history. No significant correlation, however, could be made between the patients' age, sex, or the tumor location (vestibular vs. nonvestibular schwannoma) and mutation location within the gene or type of mutation (frameshift, nonsense, splice defect, or LOH).

Distribution of NF2 Mutations in Schwannomas

The distribution of the 62 schwannoma *NF2* mutations reported here along with the distribution of 32 independent mutations that we reported previously (Jacoby et al., 1994) are presented in Figure 2. Mutations have been found in all exons except exons 16 and 17, which encode the alternative COOH-termini of the gene. Mutations were relatively evenly distributed across the first 15 exons of the gene, with no obvious hot spots. Multiple occurrences of mutations altering an arginine codon (CGA) to a stop codon (TGA) were observed for residues 57 (twice), 262 (three times), 341 (twice),

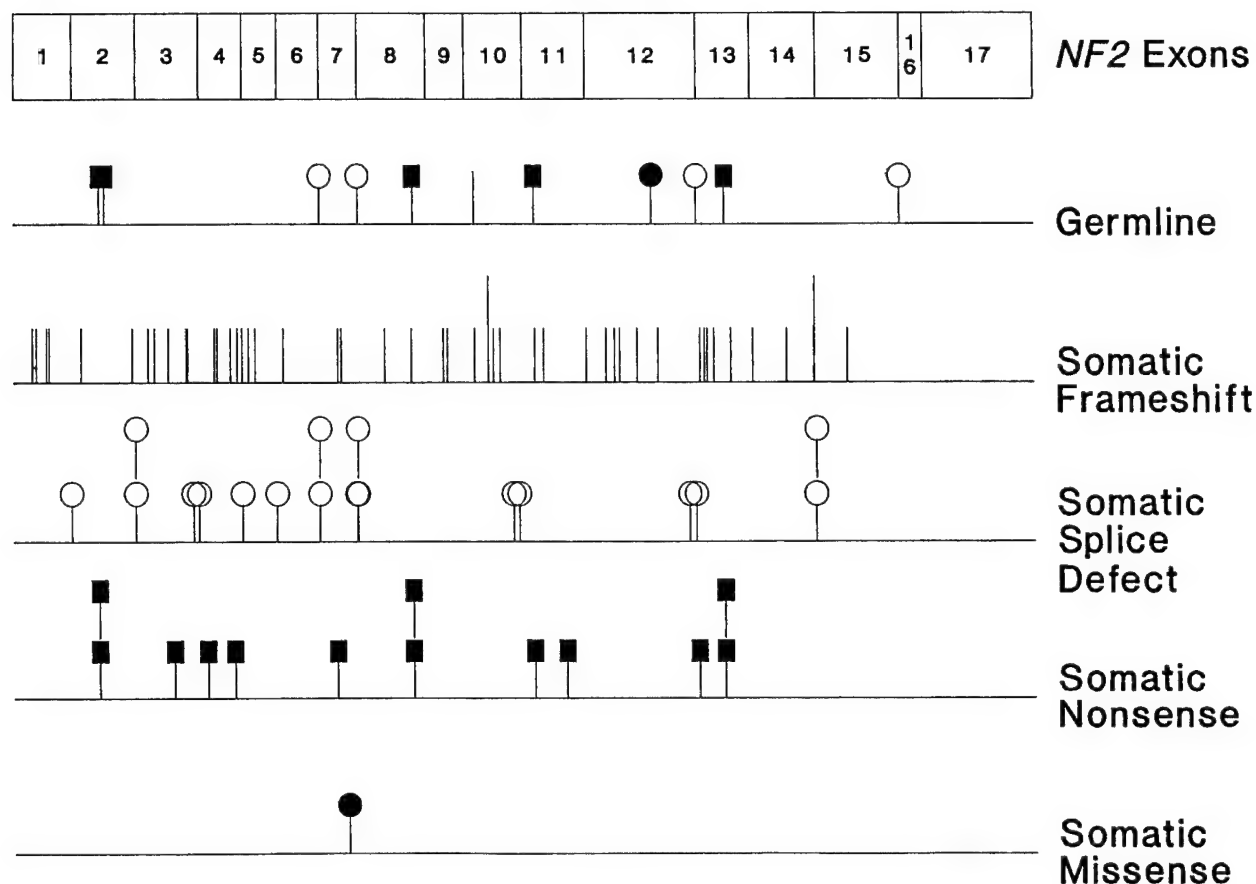


Figure 2. Distribution of *NF2* mutations in schwannomas. The figure includes 62 mutations that are listed in Tables 2 and 3 and 32 mutations reported by Jacoby et al. (1994). Distribution of 11 germline *NF2* mutations, 50 somatic frameshift mutations, 19 somatic splice mutations, 13

somatic nonsense mutations, and 1 somatic missense mutation is shown. Untopped vertical line, frameshift mutation; line topped with open circle, splice mutation; line topped with solid square, nonsense mutation; line topped with solid circle, missense mutation.

and 466 (twice). Schwannoma S114 contained two such nonsense mutations: a germline mutation in Arg341 and a somatic mutation in Arg57. The frequency of nonsense mutations at these particular CpG sites in the *NF2* gene in both germline and somatic tissues is relatively high, as noted previously (see, e.g., Sainz et al., 1995); however, no single nonsense mutation accounted for more than 5% (3 of 58) of the tumors. Along with the nonsense mutations, one frameshift mutation—a 1 bp deletion at nucleotide 932 in exon 10—was observed in two independent tumors, S38 (Jacoby et al., 1994) and S99 (this study). One splice mutation at position 600–1 was also observed in two tumors, S12 (Jacoby et al., 1994) and S119 (this study).

DISCUSSION

In this study, we report inactivating events affecting 51 of the 58 tumors examined. The high

proportion of schwannomas in which inactivating mutations were found indicates that merlin plays a fundamental role in controlling the growth of the Schwann cells that give rise to this tumor type. This study also provides evidence for complete inactivation of *NF2* in more than 60% of schwannomas, either by mutation in both alleles (15 tumors) or by mutation in one allele and loss of the other allele (23 tumors). These data strongly support the tumor suppressor model in which loss or inactivation of both copies of the *NF2* gene underlies schwannoma formation.

Alterations in the *NF2* gene were found both in the more common vestibular schwannomas and in schwannomas occurring at other peripheral sites, including the femoral nerve (S101), the fifth cranial nerve (S125), and the lumbar spinal cord (S74). A similar spectrum of mutations occurred in sporadic tumors and in patients with germline *NF2* muta-

tions. In only seven tumors, there was no alteration of the *NF2* gene found. These included five vestibular schwannomas, one thoracic spinal schwannoma, and one schwannoma located on the jugular foramen. No unique or striking clinical features were evident in these seven tumors. Given the fact that two of the tumors were from patients with a clinical diagnosis of NF2, it is likely that these tumors possessed *NF2* mutations outside the exon sequences that were scanned or that were missed by the SSCP technique, rather than mutations at an independent locus.

About two-thirds of the potential *NF2* mutations were detected by using SSCP analysis in this study. We believe that our SSCP analysis was highly efficient for the *NF2* gene, especially because we used two different electrophoretic conditions when screening for mobility shifts. Among the 63 mobility shifts detected, 55 were detected on both polyacrylamide and MDE gel matrices, five were detected only on polyacrylamide, and three were detected only on MDE gels. The remaining one-third of the mutations is likely to consist of intragenic deletions spanning primer sites or large deletions, such as those observed initially (Rouleau et al., 1993; Trofatter et al., 1993). Alternatively, they may be alterations in the noncoding intronic promoter or 3' untranslated regions.

All of the mutations reported here are predicted to result in a truncated or grossly altered merlin protein. Four germline mutations were found, and, as in previous studies (see, e.g., MacCollin et al., 1994), most of these were point mutations that created nonsense codons or that affected splicing signals. By contrast, most of the somatic mutations (40 of 58) were small deletions or, occasionally, insertions that either introduced a frameshift or interfered with normal splicing. The remaining somatic mutations (18 of 58) were point mutations that either created new stop codons or presumably disrupted splicing.

For schwannomas with predicted splicing alterations, we have provided the first direct evidence to suggest that mutations outside the *NF2* coding sequence do indeed effect gross alterations in merlin's structure. In all ten cases examined, splicing defects were confirmed by detection of aberrant cDNA products after RT-PCR. In seven cases, splice donor function was predicted to be affected, but only two of those cases resulted in the complete absence of the upstream exon from the transcript. Interestingly, in the other four tumors, activation of one or more cryptic splice sites in the upstream exon resulted in the removal of only a

portion of the exon from the transcript and introduced a frameshift, which led to premature termination that was comparable to the more common genomic deletion mutations. A similar phenomenon was observed for mutations affecting splice acceptor function, with one resulting in the activation of a cryptic splice site and two resulting in the complete absence of the downstream exon. In one exceptional case in which splice donor function was predicted to be affected, an intronic insertion actually effected the complete absence of both the upstream and the downstream exons from the transcript. These data indicate that *NF2* mutations that occur outside the coding sequence frequently cause shifts in the reading frame, even when they might have been expected to lead to in-frame skipping of a single exon. Thus, such mutations often will be predicted to produce a truncated merlin rather than a merlin with a missing internal segment.

The 94 mutations from this study and from our previous study (Jacoby et al., 1994; summarized in Fig. 2) represent more than half of those reported (Bijlsma et al., 1994; Irving et al., 1994; Lekanne Deprez et al., 1994; Sainz et al., 1994; Twist et al., 1994; Mérel et al., 1995b; Sainz et al., 1995). Those identified by other investigators are also predominantly frameshifts or point mutations that cause the truncation of merlin. However, these were not included in Figure 2, because, in most cases, the entire coding sequence together with the splice junctions was not scanned. For the schwannomas represented in Figure 2, there was no evident correlation between tumor phenotype and *NF2* genotype when clinical parameters, including patient age, patient sex, or tumor location, were paired with the type or location of the *NF2* mutations.

The distribution of *NF2* mutations indicates that merlin's tumor suppressor function can be eliminated by truncations beginning in any region of the protein except in the alternative COOH-termini encoded by exons 16 and 17. The crucial importance of both the protein 4.1 superfamily domain (exons 1–9) and the subsequent α -helical domain (exons 10–15) is also evident in the few missense mutations that have been reported to cause single amino acid substitutions in merlin. Missense mutations are rare in *NF2*, and none was detected in this study. Despite the paucity of missense mutations in schwannomas, mutation analysis has established clearly that inactivation of merlin is the critical event leading to the formation of both inherited and sporadic schwannomas. The search

for additional missense mutations in *NF2* and associated tumors is highly warranted, because such alterations offer the hope of pinpointing residues and functional domains critical for merlin's tumor suppressor activity. The delineation of merlin's normal physiologic role and of the mechanism whereby disruption of this function causes abnormal proliferation represents the next challenge to be met in increasing our understanding of schwannoma formation and in approaching the development of an effective therapy for these difficult-to-manage tumors.

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NOTE ADDED IN PROOF

The germline mutation in tumor S101 has been reported independently in another member of the same family (patient G4924, family 203) by Rutledge et al. (Am. J. Hum. Genet., in press).

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Neuropathology and Molecular Genetics of Neurofibromatosis 2 and Related Tumors

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Neurofibromatosis 2 (NF2) is an uncommon, autosomal dominant disorder in which patients are predisposed to neoplastic and dysplastic lesions of Schwann cells (schwannomas and schwannosis), meningeal cells (meningiomas and meningioangiomatosis) and glial cells (gliomas and glial hamartomas). Clinical and genetic criteria that distinguish NF2 from neurofibromatosis 1 have allowed more accurate assignment of specific pathological features to NF2. The NF2 tumor suppressor gene on chromosome 22q12 encodes a widely expressed protein, named merlin, which may link the cytoskeleton and cell membrane. Germline NF2 mutations in NF2 patients and somatic NF2 mutations in sporadic schwannomas and meningiomas have different mutational spectra, but most NF2 alterations result in a truncated, inactivated merlin protein. In NF2 patients, specific mutations do not necessarily correlate with phenotypic severity, although grossly truncating alterations may result in a more severe phenotype. In schwannomas, NF2 mutations are common and may be necessary for tumorigenesis. In meningiomas, NF2 mutations occur more commonly in fibroblastic than meningothelial subtypes, and may cluster in the first half of the gene. In addition, in meningiomas, a second, non-NF2 meningioma locus is probably also involved. Future efforts in NF2 research will be directed toward elucidating the role of merlin in the normal cell and the sequelae of its inactivation in human tumors.

Introduction

Neurofibromatosis 2 (NF2), previously known as *central neurofibromatosis* or *bilateral acoustic neurofibromatosis*, is an uncommon disorder, affecting approximately 1 in 40,000 individuals (10). The condition is inherited in an autosomal dominant manner with high penetrance, although about one half of cases have no family history and most likely represent new mutations. Patients with NF2 are genetically predisposed to a number of characteristic tumors, as well as some non-neoplastic conditions such as posterior subcapsular lens opacities. The neuropathological abnormalities are generally low-grade neoplasms or malformative conditions of Schwann cells (schwannomas and schwannosis), meningothelial cells (meningiomas and meningioangiomatosis) and glia (gliomas and glial hamartomas). The current diagnostic criteria (10, 16, 59) for NF2 are either:

1. Bilateral vestibular schwannomas; or
2. A first-degree relative with NF2, and either -- a unilateral vestibular schwannoma or -- two of the following: meningioma, schwannoma, glioma, neurofibroma, posterior subcapsular lens opacity, or cerebral calcification; or
3. Two of the following -- unilateral vestibular schwannoma -- multiple meningiomas -- either schwannoma, glioma, neurofibroma, posterior subcapsular lens opacity, or cerebral calcification.

As molecular genetic analyses of the NF2 gene become more commonplace, however, diagnostic criteria may shift, perhaps bringing a variety of NF2-variants into the fold.

The initial case reports of NF2 date to the early nineteenth century and the distinction between *peripheral neurofibromatosis* or neurofibromatosis 1 (NF1) and *central neurofibromatosis* or NF2 to the early twentieth century (see references (10, 16, 39)). In 1937 Worster-Drought et al. definitively stated that "the term von Recklinghausen's disease should be confined to the purely peripheral subcutaneous form of the disease" (72). Nonetheless, while numerous reports of NF2 have appeared in the medical literature of the past century, until recently most were lumped under the rubric *von Recklinghausen's disease*, sometimes with

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the additional designation *central* (see reference (16, 39)). The clinical and molecular genetic studies of the 1980s (23, 33, 41, 55, 57, 70), followed by the identification of the *NF2* gene in 1993 (40, 65), finally proved that NF2 is distinct from NF1 and ushered in a new era in NF2 research. As a result, NF2 and NF2-related tumors have been studied extensively in the past few years. From a pathological point of view, disease characteristics can now be assigned more definitively to NF2, thus separating NF2 lesions from what had previously been labeled *von Recklinghausen's disease* or simply *neurofibromatosis*. The discovery of the *NF2* gene has also allowed detailed analyses of the gene in NF2 patients and in NF2-associated tumors and permitted clinicopathological-genetic correlations. The following discussion reviews the major neuropathological abnormalities in NF2, particularly in relation to recent genetic data. The review also details ongoing molecular genetic evaluations into the *NF2* gene and its alterations in NF2 patients and NF2-associated tumors.

Neuropathology of NF2

Schwannoma and Schwannosis. Vestibular schwannomas (also termed *acoustic neuromas*, *neurolemmomas*, or *neurinomas*) are the primary hallmark of NF2 and, when bilateral, define a patient as having the disease. As with sporadic schwannomas, schwannomas in NF2 occur on the vestibular branch of the eighth cranial nerve and show the same predilection for the internal acoustic meatus. Typically, sensory nerves and roots, particularly the fifth cranial nerve (10) and spinal dorsal roots (13), are affected more often than primarily motor nerves, although the twelfth cranial nerve may be involved quite frequently (10). In NF2, however, schwannomas occur earlier in life, are multiple and may occur on any of the cranial and peripheral nerves. Cutaneous schwannomas occur in about half of NF2 patients, and three different variants have been described (10, 30, 59). Multiple peripheral (non-vestibular) schwannomas, however, do not currently define a patient as having NF2 unless there is also a family history of NF2 or NF2-related tumors; studies of the *NF2* gene in patients with multiple peripheral schwannomas without a family history of NF2 are ongoing, but have not yet concluded whether these patients have a variant of NF2 or a distinct disease (M.P. Short, M. MacCollin and L.B. Jacoby, personal communication).

The schwannomas that occur in NF2 are histologically similar to sporadic schwannomas, with some notable exceptions. Approximately 40% of vestibular schwannomas in NF2 tend to have a lobular, "grape-like" pattern (see Figure 1), while this pattern is extremely uncommon in sporadic schwannomas (61). Peripheral nerve schwannomas in NF2 patients may also have a distinctly multifocal appearance,

arising in multiple different locations along the same nerve. In addition, vestibular schwannomas in NF2 patients often contain embedded eighth nerve fibers, whereas embedded axons are less common in sporadic schwannomas (19). Interestingly, some of these unusual features -- particularly the lobular pattern or multifocal appearance -- may also be seen in the schwannomas from patients with multiple schwannomas who do not meet diagnostic criteria for NF2 (Figure 1). The lobular pattern most likely does not reflect a multiclonal origin for these inherited forms of schwannoma since schwannomas, including those in NF2 patients, are monoclonal tumors (21). On the other hand, the multifocal tumorlets seen along peripheral nerves in these disorders may reflect multiple independent clones, but this has not been confirmed by molecular genetic techniques.

As noted above, schwannomas in NF2 patients occur at similar sites to schwannomas in non-NF2 patients (13); notably, for instance, NF2 patients do not often develop intramedullary spinal cord schwannomas, despite the frequent presence of intramedullary schwannosis. These observations argue that additional, local factors play a role in schwannoma formation. Schwann cells from different sites may differ biologically; in this regard, it will be of interest to determine whether Schwann cells in the eighth cranial nerve differ in *NF2* gene expression from Schwann cells in nerves less susceptible to schwannoma formation. Other regional differences, such as trauma or growth factors, may also facilitate tumor formation at particular sites, as has been suggested for the preponderance of schwannomas at bony canals (38). As with sporadic schwannomas, malignant degeneration of schwannomas in NF2 is exceedingly rare (10, 23), but has been reported (71). Schwannosis is a term applied to proliferation of Schwann cells, sometimes with entangled axons, but without frank tumor formation. Schwannosis is typically noted in cases of NF2 in the spinal dorsal root entry zones, where it may be associated with a schwannoma of the dorsal root, or in the perivascular spaces of the central spinal cord, where the nodules appear more like small traumatic neuromas (43, 44, 46). Less robust, but otherwise histologically identical, schwannosis has been reported in many other conditions, probably as a reactive response to local injury (1, 24). In NF2, the sometimes exuberant nature of these microscopic foci of Schwann cell proliferation presumably reflects the underlying susceptibility of NF2 patients to abnormal Schwann cell growth. However, as discussed above, other events must be necessary for the formation of full-fledged schwannomas.

In addition to cutaneous schwannomas, typical cutaneous neurofibromas may occur in NF2 and may be multiple (10, 30, 38). Plexiform neurofibromas, however, are not seen in NF2 (10) and are instead typical

of NF1. Importantly, the presence of cutaneous neurofibromas does not necessitate a diagnosis of NF1 or mixed neurofibromatosis. Nonetheless, rare neurofibromatosis patients either meet criteria for both NF1 and NF2, or for neither, and remain nosological controversies (10, 38).

Meningioma and meningioangiomatosis. Multiple meningiomas are the second hallmark of NF2 and occur in the majority of NF2 patients. As expected for a hereditary tumor syndrome, meningiomas in NF2 tend to occur earlier in life than sporadic meningiomas and are often multiple. Patients with multiple meningiomas who lack vestibular schwannomas, however, do not meet current diagnostic criteria for NF2. Indeed, one molecular genetic study has shown that a family with multiple meningiomas but without vestibular schwannomas does not show linkage to the NF2 locus on chromosome 22q, suggesting a second, distinct meningioma predisposition gene (36). Furthermore, those patients with multiple meningiomas but without a family history of meningiomas most likely reflect multifocal spread from a single meningioma, since each tumor harbors the same NF2 gene mutation in the absence of a germline NF2 gene alteration (68).

Those meningiomas that occur in NF2 are usually histologically benign, and there is no increased incidence of either atypical or malignant meningiomas in patients with NF2. A number of authors have observed that most meningiomas in NF2 are of the fibroblastic type (46, 53), but detailed comparative studies of NF2 and non-NF2 meningiomas have not, to our knowledge, been published. Our recent review of nine meningiomas from eight NF2 patients revealed only five fibroblastic or transitional meningiomas and four meningothelial variants (M.J. Ma, M. MacCollin and D.N. Louis, unpublished observations). In sporadic meningiomas, both chromosome 22q allelic loss and NF2 gene mutations are more common in fibroblastic and transitional subtypes than in meningothelial forms (69). Combined, these histological and genetic findings may suggest that either germline or somatic inactivation of the NF2 gene results more commonly in a primarily fibroblastic, or sometimes transitional, phenotype. Interestingly, the above mentioned non-NF2 family with multiple meningiomas that did not show linkage to chromosome 22q, had strictly meningothelial meningiomas (60). Indeed, a number of other non-NF2 families with multiple meningiomas have been reported to have exclusively meningothelial meningiomas (22, 50, 54). These findings suggest that the putative second meningioma locus may be integral to the development of meningothelial subtypes, perhaps complementing the role of the NF2 locus in the fibroblastic subtypes.

Meningioangiomatosis is an uncommon condition characterized by a plaque-like proliferation of

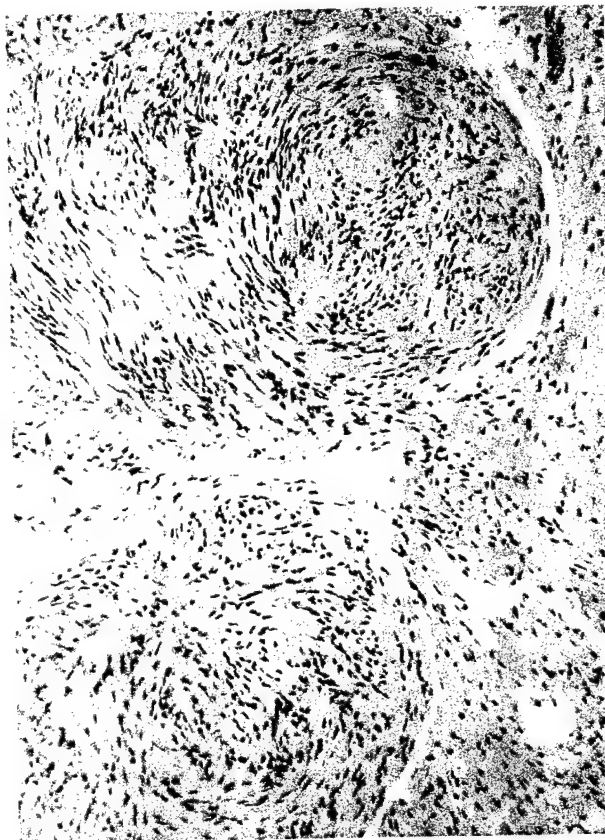


Figure 1 A "grape-like" or nodular appearance is characteristic of schwannomas in NF2 patients and in non-NF2 patients with multiple schwannomas, as illustrated in this parasagittal tumor from a non-NF2 patient with multiple schwannomas.

meningothelial and fibroblast-like cells surrounding small vessels (7, 44, 46). Meningioangiomatosis is usually a single, intracortical lesion, although multiple lesions, as well as thalamic and brain stem lesions, have been reported. The condition varies from being predominantly vascular, at times resembling a vascular malformation, to being predominantly meningothelial in nature. In addition, foci of meningioangiomatosis may be associated with the glial hamartomas described below and with neurofibrillary tangles (11, 14). The literature on meningioangiomatosis in NF has been confused by the prior lack of distinction between NF1 and NF2. A review of most reports of meningioangiomatosis suggests that the disease is strongly associated with NF2; most descriptions of meningioangiomatosis associated with *von Recklinghausen's disease* or simply *neurofibromatosis* clearly document patients with bilateral vestibular schwannomas who would now be classified as having NF2. On the other hand, reports of meningioangiomatosis in NF1 are rare (17) and not well-documented, and it is unclear whether meningioangiomatosis is biologically related to NF1. Meningioangiomatosis also occurs sporadically, and there have been numerous case reports of

"meningioma-angiomatosis not associated with von Recklinghausen's neurofibromatosis" over the past ten years (34). While the histogenesis of meningioangiomatosis remains controversial (11), most authors agree that the proliferating cells are meningeothelial (7). From this point of view, the association of meningioangiomatosis with NF2, rather than NF1, seems logical: the lesion, whether neoplastic or dysplastic, reflects the underlying tendency for meningeothelial cells to undergo proliferative changes after inactivation of the *NF2* gene. Analysis of the *NF2* gene in apparently sporadic cases of meningioangiomatosis would thus be of great interest.

Gliomas and glial hamartomas. Patients with NF2 are predisposed to glial lesions, both neoplastic and dysplastic. Gliomas in NF2, however, are less common than schwannomas and meningiomas. Approximately 80% of gliomas in NF2 patients are intramedullary spinal or cauda equina tumors, with an additional 10% of gliomas occurring in the medulla; cerebral, cerebellar and pontine gliomas are therefore uncommon complications of NF2 (39). By far the most common gliomas in patients with NF2 are intramedullary spinal ependymomas. Ependymomas account for approximately 65-75% of all histologically diagnosed gliomas in NF2, and for an even higher percentage of spinal gliomas (9, 39, 46). In most cases, NF2 patients with spinal ependymomas have multiple lesions (39, 46). To our knowledge, there have been no reported histological differences between NF2 and sporadic ependymomas, but careful correlative studies have not been performed. Ependymal heterotopias have been noted in the spinal cords of NF2 patients and, while the relationship between these heterotopias and the more commonplace glial hamartomas is unclear (see below), it has been suggested that such heterotopias may provide substrates for neoplastic transformation (46). Most types of diffuse and pilocytic astrocytomas, including optic nerve gliomas, also occur in NF2, but are less common than ependymomas and are far more characteristic of NF1.

Glial microhamartomas of the cerebral cortex are common in NF2 (46), being found in all cases in at least one series (71), but are not associated with any predisposition to mental retardation (10) or astrocytomas of the cerebral hemispheres. These hamartomas consist of circumscribed clusters of cells with medium to large, atypical nuclei and scant, sometimes stellar, eosinophilic cytoplasm (46). The cells stain strongly for S-100 protein, but only focally for glial fibrillary acidic protein. Careful studies of these cells with a wide variety of other antibodies have not suggested alternative avenues of differentiation, and most authors support the view that the hamartomatous cells are astrocytic (71). These lesions are usually intracortical, with a predilection for the molecular layer and deeper cortical layers, but have also been observed in the basal ganglia, thalamus and cerebel-

lum (71). We have also noted microscopically identical hamartomas in the dorsal horns of the spinal cord (Figure 2), where the relationship with so-called ependymal heterotopias is unclear. Indeed, since even the cerebral cortical lesions show ependymal features (46), the spinal hamartomas and ependymal heterotopias most likely represent the same condition. While the glial hamartomas of NF2 may show considerable cytological atypia, the absence of mitotic activity and of macroscopic growth, coupled with the complete lack of association between their distribution and the distribution of gliomas in NF2, make it unlikely that these are preneoplastic lesions (71).

Other neuropathological lesions. Some NF2 patients develop a mixed sensory and motor peripheral neuropathy (10, 59), which may be secondary to focal schwannomatous changes or onion bulb-like Schwann cell or perineurial cell proliferation within peripheral nerves (64). Intracranial calcifications have been noted frequently on neuroimaging studies of patients with NF2. These calcifications are not related to intracranial tumors and occur in the cerebral and cerebellar cortices, periventricular areas and choroid plexus; to our knowledge, they have not been correlated with histopathological findings (59).

Molecular Genetics of NF2 and Related Tumors

The *NF2* gene. In 1986 and 1987, molecular genetic studies of sporadic and NF2-associated schwannomas and meningiomas showed frequent allelic loss of chromosome 22, suggesting that the *NF2* gene resided on chromosome 22 (56, 57). This hypothesis was confirmed when molecular genetic analyses of large NF2 pedigrees demonstrated linkage of NF2 to chromosome 22q12 (41, 70). Subsequent analyses have shown that all studied NF2 pedigrees link to chromosome 22q, implying that defects in one gene account for all families with NF2 (32) (by contrast, see the accompanying article in this issue on tuberous sclerosis by Short et al.). Additional linkage studies were used to narrow the location of the *NF2* gene and, in 1993, the gene was cloned by two independent groups (40, 65).

The *NF2* gene spans 110 kb, comprising 16 constitutive exons and one alternatively spliced exon. NF2 mRNA transcripts occur in three different size ranges, approximately 7 kb, 4.4 kb and 2.6 kb, and encode at least two major alternative protein forms (3, 20, 40, 65). The alternatively spliced exon 16 alters the C-terminus of the protein, replacing 16 amino acids with 11 novel residues (3, 20). Additional alternative splices predicting other minor species have also been described (2, 37). The mouse homologue, which maps to mouse chromosome 11, is similarly alternatively spliced and predicts a protein that is 98% identical to human merlin (8, 12, 15).

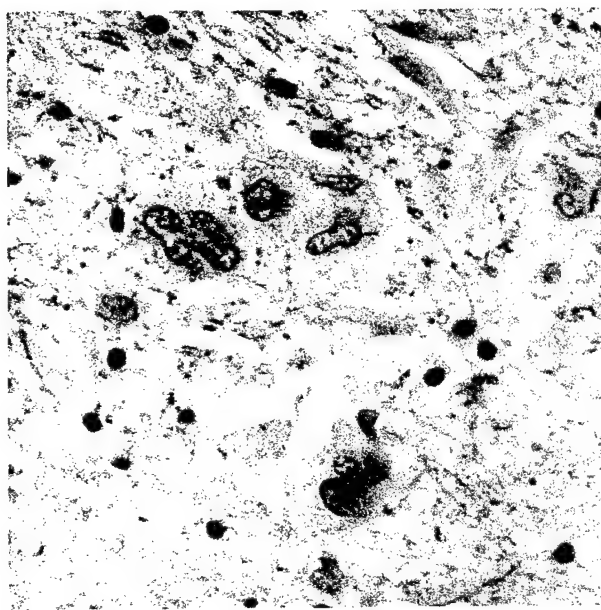


Figure 2 Large, atypical glial cells, identical to those populating cortical glial hamartomas, can also be seen in the spinal cord of NF2 patients.

The *NF2* gene is expressed in most studied normal human tissues, including heart, lung, skeletal muscle, kidney, breast, ovary and placenta, brain, pancreas and liver (40, 65). High expression has been noted in mouse fetal brain (40).

Merlin, the *NF2*-encoded protein. The predicted protein product of the *NF2* gene shows a strong similarity to the highly conserved protein 4.1 family of cytoskeleton-associated proteins, which includes protein 4.1, talin, moesin, ezrin, radixin, and two protein tyrosine phosphatases, PTP-MEG and PTP-H1. Because the *NF2*-encoded protein is most similar to moesin, ezrin and radixin, it was named *merlin*, for moesin-ezrin-radixin-like protein (65). The alternative name *schwannomin* was subsequently suggested by a second group (40). Merlin can be detected immunohistochemically in the cytoplasm of many cells, including Schwann cells (Figure 3), fibroblasts, neurons and histiocytes (unpublished data; (52), although detailed immunohistochemical studies of different human organs have been hampered to date by a lack of reliable antibodies.

While members of the protein 4.1 family probably have a number of functions, their primary role may be in mediating communication between the extracellular milieu and the cytoskeleton, by acting as a link between integral membrane proteins and the scaffolding proteins of the filamentous submembrane lattice (27). For instance, moesin, ezrin and radixin interact with CD44 as a membrane target (66). The 4.1 family proteins are defined by a homologous domain of approximately 270 amino acids

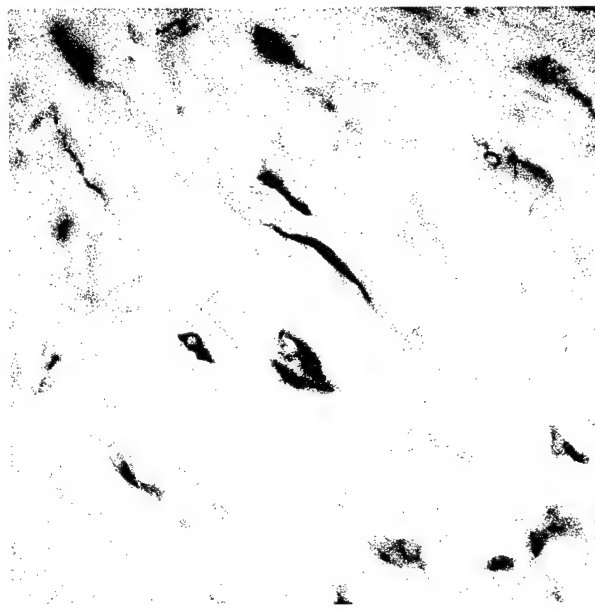


Figure 3 Immunohistochemistry with a polyclonal antibody against a merlin carboxyl-terminus peptide sequence demonstrates cytoplasmic staining of scattered Schwann cells in a frozen section of normal peripheral nerve.

near the amino-terminus (27). In merlin and its most closely related proteins, moesin, ezrin and radixin, this domain is followed by a long α -helical segment and a charged carboxyl-terminal domain. The similarity between merlin and these other three proteins suggests that merlin may also associate with both membrane and cytoskeletal structures. However, the distinct localization and behavior of moesin, ezrin and radixin make it likely that merlin also has a unique cellular role. At least five proteins, varying in size from 70 to 165 kD, appear to bind merlin at its amino-terminal moesin-ezrin-radixin homology domain (63). Since most of the described *NF2* mutations (see below) would probably alter such merlin-protein binding, these proteins may be important for merlin to effect tumor suppression.

Because the functions of merlin and its putative binding partners remain unknown, the sequelae of merlin inactivation remain speculative. Given the generally benign nature of the tumors caused by *NF2* gene mutations, merlin inactivation presumably results in an enhanced growth potential that somehow falls short of the frankly malignant transformation accompanying inactivation of other tumor suppressors, such as the Rb and p53 transcription factors. It is tempting to postulate that merlin and its binding partners participate in growth inhibitory signaling, as has been suggested for the APC tumor suppressor product binding to the cell membrane-associated proteins β -catenin and phakoglobin (42, 58, 62). In this scenario, inactivation of a merlin-mediated, cell membrane-cytoskeletal link may disrupt growth inhibitory cytoplasmic signaling from the cell surface and may thereby facilitate the neoplastic phenotype.

Table 1 Trends in mutational spectra of the NF2 gene

	Mutation	Types of mutation	Sites of mutation
NF2 patients	Germline	Point mutations > deletions	Hot spots at Arg residues ?
Schwannomas	Somatic	Deletions > point mutations	None
Meningiomas	Somatic	Deletions > point mutations	First half of coding sequence ?

Germline mutations in NF2 patients are most commonly point mutations that alter splice junctions or that create new stop codons. Somatic alterations in schwannomas and meningiomas are more commonly small deletions, or occasionally insertions, that either produce a frameshift and a premature stop codon or disrupt proper splicing. Potential hot-spots for germline mutations are in arginine codons, while somatic mutations in meningiomas may favor the first half of the coding sequence. Confirmation of these differences in mutational spectra await larger series and standardization of screening techniques.

NF2 gene mutations. Unlike the search for *NF1* gene mutations, analysis of the *NF2* gene has revealed a plethora of germline and somatic mutations that are predicted to affect protein expression, supporting the hypothesis that *NF2* functions as a tumor suppressor gene (2, 3, 5, 6, 18, 20, 28, 29, 31, 40, 45, 47, 48, 51, 52, 65, 69). These have largely been inactivating genetic alterations, such as frameshift and nonsense mutations, although rare missense mutations have also been detected. Interestingly, the types of mutations that occur in the germline of NF2 patients are somewhat different from those that occur somatically in sporadic schwannomas and meningiomas (Table 1). Point mutations that alter splice junctions or that create new stop codons are the most frequent type of germline mutation. On the other hand, somatic alterations are usually small deletions, or occasionally insertions, that either produce a frameshift and a premature stop codon or disrupt proper splicing. Furthermore, the distribution of mutations may differ between schwannomas and meningiomas, with meningioma mutations clustering in the first half of the coding sequence and schwannoma mutations dispersed throughout the gene. Confirmation of these differences in mutational spectra, however, must await larger series and standardization of screening techniques.

NF2 patients. The initial identification of merlin as the *NF2* suppressor was based on four non-overlapping interstitial deletions in four unrelated NF2 patients (65). Subsequently, a large number of additional mutations have been defined in NF2 patients (5, 6, 28, 29, 31, 40, 51, 65). Most germline mutations are predicted to truncate the protein product. As noted above, the majority of these mutations are point mutations that alter splice junctions or create new stop codons, but small and large deletions have also been documented. These alterations have been observed throughout the gene, with the exception of the alternatively spliced exons 16 and 17, but one large series has suggested that germline mutations preferentially occur in exons 1 through 8 (31). A possible hot spot for mutations may be position 169, in exon 2, in which a C to T transition at a CpG dinucleotide results in a stop at codon 57 (5, 31). Other

CpG dinucleotides are also commonly targets for C to T transitions (51), presumably from deamination of 5-methylcytosine to thymine, as has been documented in other tumor suppressor genes such as p53 (26). In the *NF2* gene, these transitions typically convert an arginine to a stop codon (51).

Some authors have suggested that there are two phenotypes for NF2: the milder "Gardner" phenotype, in which patients develop bilateral vestibular schwannomas later in life without many other nervous system tumors, and the severe "Wishart" phenotype, in which multiple meningiomas and ependymomas accompany early bilateral vestibular schwannomas (10). Since all families with NF2 link to chromosome 22 (32), implying a single responsible gene, the phenotypic subtypes raise the possibility of allelic variants of NF2. However, while most families display relative homogeneity, "Wishart" and "Gardner" phenotypes can occur in the same families, making the possibility of allelic variants less likely. Recently, two unrelated NF2 patients, one with the severe "Wishart" and the other with the mild "Gardner" phenotype, have been reported to have the same constitutional *NF2* mutation (6). This observation suggests that factors other than the specific *NF2* mutation must regulate phenotypic expression of a mutant *NF2* gene. One such factor may be somatic mosaicism for the *NF2* mutation. Somatic mosaicism occurs when a mutation occurs early in embryogenesis rather than in the germline; as a result, only some cells bear the mutation. In the above-mentioned report of two phenotypically dissimilar patients with the same *NF2* mutation, the patient with the mild "Gardner" phenotype was mosaic for the *NF2* mutation (6). On the other hand, one recent study has suggested that patients with milder phenotypes are associated with mutations which preserve the carboxyl-terminus of the protein, while grossly truncating mutations result in the more severe phenotype (31). Further correlative analyses of NF2 phenotype and genotype will be necessary to clarify the complicated and perhaps multifactorial issue of phenotypic variation.

Schwannomas. *NF2* gene mutations have now been detected in numerous schwannomas, confirming the prediction that this tumor suppressor is integral to

schwannoma formation (4, 18, 20, 25, 51, 52, 67). Most studies have identified mutations in at least 50% of schwannomas, in vestibular tumors as well as schwannomas from other sites. For instance, our initial study of the entire coding region of the *NF2* gene found mutations in 19 of 30 sporadic schwannomas (20). Our continued analysis of a second cohort of 60 vestibular schwannomas using combined screening methods, however, has revealed an additional 63 mutations. These recent findings suggest that *NF2* mutations may be present in the vast majority of schwannomas (L.B. Jacoby, personal communication). The majority of the somatic changes are small deletions or insertions that create either frameshifts and premature stop codons or altered splicing. Inactivating mutations have been detected in all exons except exons 16 and 17, which encode the alternative carboxyl-termini, and are relatively evenly distributed across the first 15 exons with no outstanding hot spots. In schwannomas, germline *NF2* mutations in NF2 patients and somatic *NF2* mutations in sporadic schwannomas are often accompanied by allelic loss of the other chromosome 22q, in accordance with the "two-hit" model of tumor suppressor gene inactivation. Thus, inactivation of *NF2* is a common feature underlying both inherited and sporadic forms of schwannoma. One study has confirmed such inactivation at the protein level, showing loss of merlin expression by immunohistochemistry in schwannomas (52).

Meningiomas. The *NF2* gene has also been studied in sporadic meningiomas, although these studies have been fewer than those addressing the *NF2* gene in schwannomas (25, 48, 69). Nonetheless, these investigations have detected *NF2* gene mutations in numerous meningiomas, thus clearly implicating this gene in meningotheial tumorigenesis. As in schwannomas, *NF2* gene alterations result predominantly in immediate truncation, splicing abnormalities or altered reading frames. Interestingly, the two large studies of the entire *NF2* gene in meningiomas showed a clustering of mutations in the moesin-ezrin-radixin homology domain in the first half of the coding sequence (25, 69). *NF2* mutations in meningiomas are highly associated with allelic loss of chromosome 22, supporting the view that the *NF2* gene represents the purported meningioma locus on this chromosome.

As mentioned above, the majority of fibroblastic meningiomas have *NF2* gene mutations and allelic loss of chromosome 22q, but a minority of meningotheial tumors show these alterations. As a result, estimates of all meningiomas suggest that approximately 40% of all meningiomas have neither *NF2* gene mutations nor allelic loss of chromosome 22q. For these tumors, it is likely that a second meningioma tumor suppressor gene is involved. This

putative second gene is probably not on chromosome 22q, since *NF2* gene mutations in meningiomas correlate closely with chromosome 22q loss. Nonetheless, a few meningiomas have been described with loss of portions of chromosome 22q that do not include the *NF2* gene, suggesting the possibility of a second meningioma locus on chromosome 22 (49). One candidate gene from this second chromosome 22q region is *BAM22*, a member of the β -adaptin gene family, which may be inactivated in some sporadic meningiomas (35). In addition, as discussed above, the putative familial meningioma gene may also be this second meningiomas locus (see Neuropathology section above).

Other tumors. The observations that NF2 patients are predisposed to spinal ependymomas and that chromosome 22q loss occurs in sporadic ependymomas, suggest that the *NF2* gene plays a role in ependymoma tumorigenesis. We studied the entire coding region of the *NF2* gene in eight ependymomas and found only a single mutation, in a spinal ependymoma that had lost the other copy of chromosome 22q (45). Therefore, while the *NF2* gene probably functions as a tumor suppressor in some ependymomas, a second chromosome 22q ependymoma suppressor gene remains a possibility. In astrocytomas, which also show allelic loss of chromosome 22q and which occur in patients with NF2, extensive analyses have failed to implicate the *NF2* gene (45). Preliminary evidence also suggests that *NF2* mutations may occur rarely non-NF2 related tumors, such as melanoma, breast cancer and colorectal cancer (2, 3, 47). Finally, in the long-standing debate over whether intracranial hemangiopericytomas are subtypes of meningiomas, *NF2* gene analysis supports the prevalent clinical, immunohistochemical and ultrastructural impression that hemangiopericytomas are not biologically related to meningiomas (J. Joseph and D.N. Louis, in preparation).

Conclusions

Recent clinical, pathological and genetic studies have delineated the characteristic features of NF2 and the salient neuropathological and molecular genetic findings have been summarized above. These seminal studies have also opened up new vistas for further discoveries. The interesting nature of this tumor suppressor and the existence of closely related family members suggest that detailed investigations of merlin function could provide fascinating new insights into cellular signaling, membrane remodeling, cytoskeletal changes, control of cell shape, and regulation of cell growth.

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Neurofibromatosis 2: loss of merlin's protective spell

James F Gusella, Vijaya Ramesh, Mia MacCollin and Lee B Jacoby

Schwannomas and meningiomas occur as multiple tumors in sufferers of neurofibromatosis 2 (NF2) and as solitary tumors in the general population due to the inactivation of a gene at chromosome 22q12. In 1993, a location cloning approach revealed this tumor suppressor, dubbed merlin, as a novel member of a family of proteins that link elements of the cytoskeleton and the cell membrane. Subsequent investigations have confirmed merlin's role in tumor formation, but have yet to reveal its mechanism of action.

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Abbreviations

ERM ezrin, radixin and moesin
merlin moesin/ezrin/radixin-like protein
NF2 neurofibromatosis 2
RT-PCR reverse transcriptase polymerase chain reaction

Introduction

Neurofibromatosis 2 (NF2) (also known as central neurofibromatosis or bilateral acoustic neurofibromatosis) is a severe inherited disorder that is genetically and clinically distinct from the more common neurofibromatosis 1 (NF1) (also known as von Recklinghausen neurofibromatosis or peripheral neurofibromatosis) [1,2]. NF2 is inherited in an autosomal dominant fashion with a high penetrance and affects approximately 1 in 40 000 individuals, with about one half of NF2 cases lacking a family history and therefore thought to be new mutations [3]. The cardinal feature of NF2 is the bilateral occurrence of vestibular schwannomas (formerly called acoustic neuromas)—benign, slow-growing tumors on the vestibular branch of the eighth cranial nerve—but meningiomas and spinal schwannomas are also frequent. In addition, many patients develop posterior capsular lens opacities [4,5]. Although the tumors associated with NF2 typically occur as multiple separate growths in the afflicted individual, sporadic, solitary tumors of the same type occur frequently in the general population. Individuals with NF2 usually develop symptoms early in adulthood, but disease-onset may occur anytime from childhood to late in life. Although benign, NF2 tumors are very difficult to manage. Compression of adjacent nerves by tumor growth causes functional impairment, including deafness and balance disorder, and surgical removal often risks permanent nerve damage. Consequently, NF2 patients suffer significant morbidity, having a significantly shortened average life span as a result.

In the period 1986-1987, analyses of polymorphic DNA markers revealed loss of heterozygosity on chromosome 22 as a frequent event both in the tumors of NF2 and in their sporadic counterparts [6-8]. These studies predicted that the *NF2* gene lay on chromosome 22 and that it encoded 'tumor suppressor' that acted normally to prevent tumor growth. In the tumor suppressor model [9], both copies of the gene must be inactivated for a cell to escape this step in growth control and consequently to form a tumor. Members of families predisposed to NF2, where an inactivating mutation in the tumor suppressor gene is transmitted through the germ-line, suffer multiple growths because a single somatic mutation in any target cell is sufficient to eliminate the remaining allele and initiate tumor development. By contrast, in sporadic cases, afflicted individuals usually develop just a single growth because of the rarity with which two different somatic mutation events inactivate both tumor suppressor alleles in any one target cell. Genetic linkage analysis in a large family predisposed to NF2 confirmed that the disease gene maps to chromosome 22, and prompted a location cloning effort to determine the nature of the tumor suppressor protein [10]. The search was completed in early 1993 when a candidate gene was identified, with a predicted protein product that displayed a strong similarity to a family of cytoskeleton-associated proteins, and which was consequently named merlin (for moesin-ezrin-radixin-like protein)[11].

The *NF2* gene and its mutations

It is now known that merlin is encoded by the *NF2* gene which spans 110 kb, comprising 16 constitutive exons and one alternatively spliced exon [11-14,15*]. *NF2* is expressed in wide variety of cell types from different tissues, producing mRNAs in three different size ranges, 7 kb, 4.4 kb and 2.6 kb, that encode at least two major alternative forms of merlin [11,16]. Isoform 1 is a protein of 595 amino acids produced from exons 1-15 and 17. The presence of the alternatively spliced exon 16 alters the carboxyl terminus of the protein, replacing 16 amino acids with 11 novel residues in isoform 2. Additional alternative splices that give rise to other minor species have also been described [17,18]. Merlin appears highly conserved through evolution; the mouse protein is 98% identical to human merlin and the mouse *Nf2* gene, which maps to chromosome 11 in a region of synteny conservation with chromosome 22q, is similarly alternatively spliced [19-23]. Strict constraints on the primary sequence of merlin are also implied by the lack of any frequent polymorphisms in the coding sequence of the *NF2* gene, even in third base codon positions [15*,24*].

The initial identification of merlin as the *NF2* suppressor was based on four non-overlapping interstitial deletions in cells or tumors of four independent *NF2* patients [11]. Later, an independent cloning effort [12] implicated the same protein (given a second name, schwannomin) as the *NF2* tumor suppressor based on more than a dozen truncating mutations in *NF2* patients and *NF2*-related tumors [12]. In the past two years, overwhelming evidence has been obtained suggesting that inactivation of merlin causes tumor development in incidences of *NF2* and their sporadic counterparts.

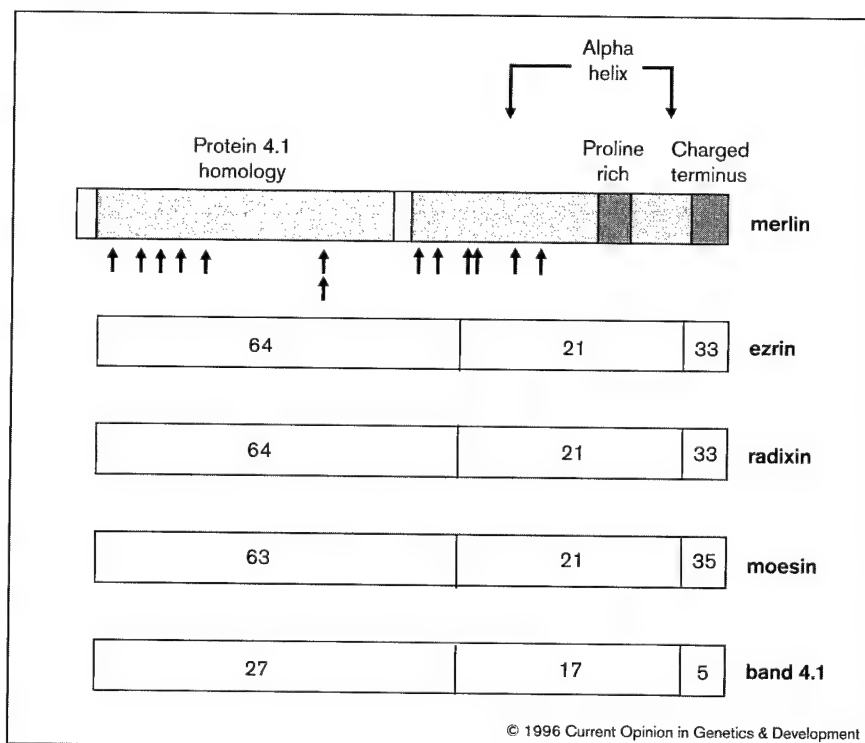
Studies aimed at identifying germline mutations in *NF2* patients, either by RT-PCR of merlin mRNA or more often by scanning exons amplified from genomic DNA, have met with great success [24,25–27,28,29–35]. A wide variety of mutations have been identified in all *NF2* exons, except exons 16 and 17. The vast majority of the alterations suggest truncation of merlin—either as introduction of a stop codon, a frameshift with premature termination, or a splicing alteration—supporting the view that loss of the protein's normal function is crucial to the development of tumors. In-frame deletions and missense mutations have also been found, however, indicating that alteration of particular functional domains can abolish merlin's tumor-suppressor activity (Fig. 1). No clear correlation between phenotype and genotype has emerged from the mutational analysis of *NF2* patients

[28], although it has been suggested that patients with non-truncating mutations may often display less severe symptoms [34].

Somatic mutations of *NF2* have also been sought, and found with relative ease, in both *NF2* and sporadic schwannomas and meningiomas [14,15,36–38,39,40,41–44]. In several cases, two different mutations have been found in the same tumor, whereas in many others only a single mutation has been detected, combined with loss of the normal allele, as revealed by loss of heterozygosity for adjacent polymorphic markers. Both results provide strong support for the tumor-suppressor model, although some evidence suggests that a minority of sporadic meningiomas do not possess mutations in *NF2* and are probably caused by mutations elsewhere in the genome. As with the germline *NF2* mutations, almost all of the somatic mutations in schwannomas and meningiomas tumors are predicted to truncate the protein product. Somatic alterations most frequently occur as small deletions (or occasionally insertions) that either produce a frameshift leading to a premature stop codon or are predicted to disrupt proper splicing. Point mutations that alter splice junctions, create new stop codons, or that produce an amino acid substitution are more frequent among germline mutations, suggesting that different mutational mechanisms may predominate at different developmental stages.

Figure 1

Merlin, a tumor suppressor related to the ERM proteins. This schematic diagram depicts merlin's domains, which include the following: a region of homology in the amino-terminal half that defines membership in the protein 4.1 family; a long α -helical domain in the second half, which is interrupted by a proline-rich stretch; and a charged carboxyl terminus. Arrows below the diagram indicate the relative locations of 13 merlin frame-preserving mutations involving either a missense change causing an amino acid substitution or a codon deletion removing a single residue, found in schwannomas, meningiomas, colon carcinomas or *NF2* patients. Comparable diagrams are shown for the ERM proteins and protein 4.1, dividing the proteins into three regions on the basis of their degree of amino acid identity with merlin. The percent amino acid identity is shown within each region.



The analyses of germline and somatic mutations leave little doubt that merlin is a tumor suppressor, the loss of which results in the formation of most schwannomas and meningiomas, and that both the amino-terminal and carboxy-terminal halves of merlin (see below) are crucial for function. Even so, the presence in normal cells of minor transcripts missing individual exons, combined with the occasional expression of these transcripts in tumor cells, suggests that some isoforms of merlin may not have tumor-suppressing ability. It remains unclear whether only one or both of the two major merlin isoforms have tumor-suppressor capacity, but the absence of tumor-producing mutations in exons 16 and 17 suggests that neither of the alternative carboxy termini of merlin, is by itself, essential for this particular function.

Loss of heterozygosity has also been observed for chromosome 22q markers in many different types of tumor not characteristically associated with NF2. Screening for mutations that affect merlin in such tumors has yielded mixed results. Only a handful of putative mutations of the *NF2* gene have been found in malignant melanoma, breast adenocarcinoma and colon cancer and none has been seen in ovarian carcinoma, hepatocellular carcinoma and astrocytoma, suggesting that merlin is not the 22q tumor suppressor frequently involved in any of these tumor types [13,14,45–48,49•]. On the other hand, two recent reports [49•,50•] demonstrate a high rate of *NF2* mutation in malignant mesothelioma, suggesting that loss of merlin may be an important step in the progression of this aggressive mesodermal tumor type [49•,50•]. Thus, merlin appears to play an important, if not primary, role in the proliferation of some cells other than those giving rise to tumors of the central nervous system.

merlin protein and its relatives

Although the identity of merlin as the NF2 tumor suppressor is now established, the protein's normal physiological role and its mechanism of action as a tumor suppressor are not. Merlin's sequence places it in the protein 4.1 family of cytoskeleton-associated proteins (Fig. 1). The members of this family, protein 4.1, talin, ezrin, radixin, moesin, merlin, and several protein tyrosine phosphatases, are defined by a homologous domain of ~270 amino acids near the amino terminus [51]. In merlin and these related proteins, this domain is followed by a long α -helical segment and a charged carboxy terminal domain. Protein 4.1, the best characterized member of the family, plays a critical role in maintaining membrane stability and cell shape in the erythrocyte by connecting integral membrane proteins, glycophorin and the anion channel, to the spectrin-actin lattice of the cytoskeleton. Genetic defects in protein 4.1 are associated with one form of hereditary elliptocytosis [52,53].

Even though its overall structure is similar to protein 4.1, merlin's sequence is more closely related to three

other members of the family: ezrin, radixin and moesin (ERM) proteins. All four proteins are predicted to have similar structural domains, although they show much higher sequence identity in the amino-terminal protein 4.1 type globular domain than in the ensuing α -helical segment. Ezrin, radixin and moesin share a 70–75% amino acid identity with each other, whereas merlin is more distantly related, displaying a 45–47% identity with each of the ERM proteins. Merlin is the only one of these four proteins reported to have alternative carboxy termini.

Ezrin is the most comprehensively studied of the ERM proteins, having first been described as a minor component of intestinal brush border microvilli and subsequently found to be identical to several proteins isolated from actin complexes of the cytoskeleton, such as cytovillin from the microvilli of choriocarcinoma cells, 80K phosphoprotein from induced microvilli of parietal cells, and p81 protein from A431 epidermoid carcinoma cells [54]. Ezrin is expressed in a wide variety of cultured cells, where it co-localizes with actin at cell surface cytoskeletal structures, and is also present in the cytoplasm [54]. Like protein 4.1, ezrin associates with the membrane via its amino-terminal half, and with the cytoskeleton via its carboxy-terminal half. Radixin was isolated from the cell-cell adherens junction (in this case from the mouse liver, [55]), where it is proposed to cap actin filaments and facilitate their attachment to the cell membrane. Moesin was originally proposed to function as a receptor for heparin sulfate, but was subsequently recognized as a component of the submembrane complex underlying filopodia and other cell surface protrusions [56]. Thus, the ERM proteins are components of actin-rich cell surface projections, such as microvilli, membrane ruffles, and filopodia and may be required for organizing, forming or maintaining these structures [57•]. All three proteins are thought to link the cytoskeletal lattice to the cell membrane, a view supported by the recent finding that the extracellular matrix receptor CD44 is an integral membrane protein target of the ERM proteins [58•].

The similarity between merlin and the ERM proteins suggests that merlin may also associate with both membrane and cytoskeletal structures and evidence from initial immunohistochemical studies support localization of merlin at or near the cell membrane [59]. Merlin might thus play a role in the control of cell shape, movement, attachment, communication, or membrane remodeling. Even so, none of the ERM proteins has yet been implicated directly in growth regulation, suggesting that merlin may also have an additional critical interaction with a signal transduction pathway which, when disrupted, leads to tumor formation. The existence of several protein tyrosine phosphatases with protein 4.1 family domains that presumably lead them to cell membrane targets may provide examples of similar localized proteins that participate in signal transduction [60–62].

Merlin might function in a pathway designed to convey information between the cytoskeleton and the cell membrane, and from these to the nucleus. The importance of this pathway in growth regulation is most evident in Schwann cells and meningeal cells, and only secondary, or redundantly controlled, in those cell types that do not give rise to frequent tumors in NF2. Preliminary evidence has been gathered that merlin overexpression can inhibit cell growth [63] and can interfere with Ras-mediated transformation [64] in mouse fibroblasts, but the biological significance of these phenomena in relation to NF2 is not known. Ultimately, it must also be considered that each of merlin's isoforms might have an array of functional characteristics, only some of which may be crucial to merlin's capacity as a tumor suppressor. For example, isoform 1 of merlin has already been reported to bind to at least five, thus far, anonymous proteins in neuroblastoma cells [65], suggesting that a wealth of protein-protein interactions may have to be investigated to identify those that are of specific importance to NF2.

Conclusions

The authentication of merlin as the tumor suppressor associated with NF2 has opened a new window on the possible mechanisms involved in growth control by suggesting that membrane-cytoskeleton interactions may be of a central importance in certain cell types. The next step is to define merlin's normal function. The coming year should see considerable progress on a number of fronts, including the following: development of better immunological reagents to specifically visualize merlin and its domains; a better delineation of merlin's subcellular localization relative to the existing ERM proteins; examination of merlin, and creation of inactivating mutations, in model organisms such as *Drosophila* and the mouse; and the characterization of some of merlin's targets of interaction.

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